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| (54) Title: GENES OF CAROTENOID BIOSYNTHESIS AND METABOLISM AND A SYSTEM FOR SCREENING FOR SUCH GENES | | |
| (57) Abstract The present invention also describes the DNA sequence for eukaryotic genes encoding ϵ cyclase, isopentenyl pyrophosphate isomerase and β -carotene hydroxylase as well as vectors containing the same and hosts transformed with said vectors. The present invention provides methods for controlling the ratio of various carotenoids in a host and for the production of novel carotenoid pigments. The present invention also provides a method for screening for eukaryotic genes encoding carotenoid biosynthesis. | | |

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TITLE OF THE INVENTION

GENES OF CAROTENOID BIOSYNTHESIS AND METABOLISM
AND A SYSTEM FOR SCREENING FOR SUCH GENES

BACKGROUND OF THE INVENTIONField of the Invention

The present invention describes the DNA sequence for eukaryotic genes encoding ϵ cyclase, isopentenyl pyrophosphate isomerase (IPP) and β -carotene hydroxylase as well as vectors containing the same and hosts transformed with said vectors. The present invention also provides a method for augmenting the accumulation of carotenoids and production of novel and rare carotenoids. The present invention provides methods for controlling the ratio of various carotenoids in a host. Additionally, the present invention provides a method for screening for eukaryotic genes encoding enzymes of carotenoid biosynthesis and metabolism.

Discussion of the Background

Carotenoid pigments with cyclic endgroups are essential components of the photosynthetic apparatus in oxygenic photosynthetic organisms (e.g., cyanobacteria, algae and plants; Goodwin, 1980). The symmetrical bicyclic yellow carotenoid pigment β -carotene (or, in rare cases, the asymmetrical bicyclic α -carotene) is intimately associated with the photosynthetic reaction centers and plays a vital role in protecting against potentially lethal photooxidative damage (Koyama, 1991). β -carotene and other carotenoids

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derived from it or from α -carotene also serve as light-harvesting pigments (Siefermann-Harms, 1987), are involved in the thermal dissipation of excess light energy captured by the light-harvesting antenna (Demmig-Adams & Adams, 1992), provide substrate for the biosynthesis of the plant growth regulator abscisic acid (Rock & Zeevaart, 1991; Parry & Horgan, 1991), and are precursors of vitamin A in human and animal diets (Krinsky, 1987). Plants also exploit carotenoids as coloring agents in flowers and fruits to attract pollinators and agents of seed dispersal (Goodwin, 1980). The color provided by carotenoids is also of agronomic value in a number of important crops. Carotenoids are currently harvested from plants for use as pigments in food and feed.

The probable pathway for formation of cyclic carotenoids in plants, algae and cyanobacteria is illustrated in Figure 1. Two types of cyclic endgroups are commonly found in higher plant carotenoids, these are referred to as the β and ϵ cyclic endgroups (Fig. 3.; the acyclic endgroup is referred to as the ψ or psi endgroup). These cyclic endgroups differ only in the position of the double bond in the ring. Carotenoids with two β rings are ubiquitous, and those with one β and one ϵ ring are common, but carotenoids with two ϵ rings are rarely detected. β -Carotene (Fig. 1) has two β endgroups and is a symmetrical compound that is the precursor of a number of other important plant carotenoids such as zeaxanthin and violaxanthin (Fig. 2).

Carotenoid enzymes have previously been isolated from a variety of sources including bacteria (Armstrong et al., 1989, Mol. Gen. Genet. 216, 254-268; Misawa et al., 1990, J. Bacteriol., 172, 6704-12), fungi (Schmidhauser et al., 1990, Mol. Cell. Biol. 10, 5064-70), cyanobacteria (Chamovitz et al., 1990, Z. Naturforsch., 45c, 482-86) and higher plants (Bartley et al., Proc. Natl. Acad. Sci USA 88, 6532-36; Martinez-Ferez & Vioque, 1992, Plant Mol. Biol. 18, 981-83). Many of the isolated enzymes show a great diversity in function and inhibitory properties between sources. For example, phytoene desaturases from *Synechococcus* and higher plants carry out a two-step desaturation to yield β -carotene as a reaction product; whereas the same enzyme from *Erwinia* introduces four double bonds forming lycopene. Similarity of the amino acid sequences are very low for bacterial versus plant enzymes. Therefore, even with a gene in hand from one source, it is difficult to screen for a gene with similar function in another source. In particular, the sequence similarity between prokaryotic and eukaryotic genes is quite low.

Further, the mechanism of gene expression in prokaryotes and eukaryotes appears to differ sufficiently such that one can not expect that an isolated eukaryotic gene will be properly expressed in a prokaryotic host.

The difficulties in isolating related genes is exemplified by recent efforts to isolate the enzyme which catalyzes the formation of β -carotene from the acyclic precursor lycopene. Although this enzyme had been isolated in a prokaryote, it had not been isolated from any photosynthetic organism nor had the corresponding genes been identified and sequenced or the cofactor requirements established. The isolation and characterization of the enzyme catalyzing formation of β -carotene in the cyanobacterium *Synechococcus* PCC7942 was described by the present inventors and others (Cunningham et al., 1993 and 1994).

The need remains for the isolation of eukaryotic genes involved in the carotenoid biosynthetic pathway, including a gene encoding an ϵ cyclase, IPP isomerase and β -carotene hydroxylase. There remains a need for methods to enhance the production of carotenoids. There also remains a need in the art for methods for screening for eukaryotic genes encoding enzymes of carotenoid biosynthesis and metabolism.

SUMMARY OF THE INVENTION

Accordingly, a first object of this invention is to provide isolated eukaryotic genes which encode enzymes involved in carotenoid biosynthesis; in particular, ϵ cyclase, IPP isomerase and β -carotene hydroxylase.

A second object of this invention is to provide eukaryotic genes which encode enzymes which produce novel carotenoids.

A third object of the present invention is to provide vectors containing said genes.

A fourth object of the present invention is to provide hosts transformed with said vectors.

Another object of the present invention is to provide hosts which accumulates novel or rare carotenoids or which overexpress known carotenoids.

Another object of the present invention is to provide hosts with inhibited carotenoid production.

Another object of this invention is to secure the expression of eukaryotic carotenoid-related genes in a recombinant prokaryotic host.

A final object of the present invention is to provide a method for screening for eukaryotic genes which encode enzymes involved in carotenoid biosynthesis and metabolism.

These and other objects of the present invention have been realized by the present inventors as described below.

BRIEF DESCRIPTION OF THE DRAWINGS

A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the

following detailed description when considered in connection with the accompanying drawings, wherein:

Figure 1 is a schematic representation of the pathway of β -carotene biosynthesis in cyanobacteria, algae and plants. The enzymes catalyzing various steps are indicated at the left. Target sites of the bleaching herbicides NFZ and MPTA are also indicated at the left. Abbreviations: DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; LCY, lycopene cyclase; MVA, mevalonic acid; MPTA, 2-(4-methylphenoxy)triethylamine hydrochloride; NFZ, norflurazon; PDS, phytoene desaturase; PSY, phytoene synthase; ZDS, ζ -carotene desaturase; PPPP, prephytoene pyrophosphate.

Figure 2 depicts possible routes of synthesis of cyclic carotenoids and common plant and algal xanthophylls (oxycarotenoids) from neurosporene. Demonstrated activities of the β - and ϵ -cyclase enzymes of *A. thaliana* are indicated by bold arrows labelled with β or ϵ respectively. A bar below the arrow leading to ϵ -carotene indicates that the enzymatic activity was examined but no product was detected. The steps marked by an arrow with a dotted line have not been specifically examined. Conventional numbering of the carbon atoms is given for neurosporene and α -carotene. Inverted triangles (∇) mark positions of the double bonds introduced as a consequence of the desaturation reactions.

Figure 3 depicts the carotene endgroups which are found in plants.

Figure 4 is a DNA sequence and the predicted amino acid sequence of ϵ cyclase isolated from *A. thaliana* (SEQ ID NOS: 1 and 2). These sequences were deposited under Genbank accession number U50738. This cDNA is incorporated into the plasmid pATeps.

Figure 5 is a DNA sequence encoding the β -carotene hydroxylase isolated from *A. thaliana* (SEQ ID NO: 3). This cDNA is incorporated into the plasmid pATOHb.

Figure 6 is an alignment of the predicted amino acid sequences of *A. thaliana* β -carotene hydroxylase (SEQ ID NO: 4) with the bacterial enzymes from *Aliccalgenes sp.* (SEQ ID NO: 5) (Genbank D58422), *Erwinia herbicola* Eho10 (SEQ ID NO.: 6) (GenBank M872280), *Erwinia uredovora* (SEQ ID NO.: 7) (GenBank D90087) and *Agrobacterium aurianticum* (SEQ ID NO.: 8) (GenBank D58420). A consensus sequence is also shown. Consensus is identical for all five genes where a capital letter appears. A lowercase letter indicates that three of five, including *A. thaliana*, have the identical residue. TM; transmembrane

Figure 7 is a DNA sequence of a cDNA encoding an IPP isomerase isolated from *A. thaliana* (SEQ ID NO: 9). This cDNA is incorporated into the plasmid pATDP5.

Figure 8 is a DNA sequence of a second cDNA encoding another IPP isomerase isolated from *A. thaliana* (SEQ ID NO: 10). This cDNA is incorporated into the plasmid pATDP7.

Figure 9 is a DNA sequence of a cDNA encoding an IPP isomerase isolated from *Haematococcus pluvialis* (SEQ ID NO: 11). This cDNA is incorporated into the plasmid pHP04.

Figure 10 is a DNA sequence of a second cDNA encoding another IPP isomerase isolated from *Haematococcus pluvialis* (SEQ ID NO: 12). This cDNA is incorporated into the plasmid pHP05.

Figure 11 is an alignment of the predicted amino acid sequences of the IPP isomerase isolated from *A. thaliana* (SEQ ID NO.: 16 and 18), *H. pluvialis* (SEQ ID NOS.: 14 and 15), *Clarkia breweri* (SEQ ID NO.: 17) (See, Blanc & Pichersky, Plant Physiol. (1995) 108:855; Genbank accession no. X82627) and *Saccharomyces cerevisiae* (SEQ ID NO.: 19) (Genbank accession no. J05090).

Figure 12 is a DNA sequence of the cDNA encoding an IPP isomerase isolated from marigold (SEQ ID NO: 13). This cDNA is incorporated into the plasmid pPMDP1. xxx's denote a region not yet sequenced at the time when this applicaiton was prepared.--

Figure 13 is an alignment of the consensus sequence of 4 plant β -cyclases (SEQ ID NO.: 20) with the *A. thaliana* ϵ -cyclase (SEQ ID NO.: 21) A capital letter in the plant β consensus is used where all 4 β cyclase genes predict the same amino acid residue in this position. A small letter indicates that an identical residue was found in 3 or the 4. Dashes indicate that the amino acid residue was not conserved and

dots in the sequence denote a gap. A consensus for the aligned sequences is given, in capital letters below the alignment, where the β and ϵ cyclase have the same amino acid residue. Arrows indicate some of the conserved amino acids that will be used as junction sites for construction of chimeric cyclases with novel enzymatic activities. Several regions of interest including a sequence signature indicative of a dinucleotide-binding motif and 2 predicted transmembrane (TM) helical regions are indicated below the alignment and are underlined.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Isolated eukaryotic genes which encode enzymes involved in carotenoid biosynthesis

The present inventors have now isolated eukaryotic genes encoding ϵ cyclase and β -carotene hydroxylase from *A. thaliana* and IPP isomerases from several sources.

The present inventors have now isolated the eukaryotic gene encoding the enzyme IPP isomerase which catalyzes the conversion of isopentenyl pyrophosphate (IPP) to dimethylallyl pyrophosphate (DMAPP). IPP isomerases were isolated from *A. thaliana*, *H. pluvialis* and marigold.

Alignments of these are shown in Figure 12 (excluding the marigold sequence). Plasmids containing these genes were deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville MD 20852 on March 4, 1996 under ATCC

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accession numbers 98000 (pHP05 - *H. pluvialis*); 98001 (pMDP1 - marigold); 98002 (pATDP7 - *H. pluvialis*) and 98004 (pHP04 - *H. pluvialis*).

The present inventors have also isolated the gene encoding the enzyme, ϵ cyclase, which is responsible for the formation of ϵ endgroups in carotenoids. A gene encoding an ϵ cyclase from any organism has not heretofore been described. The *A. thaliana* ϵ cyclase adds an ϵ -ring to only one end of the symmetrical lycopene while the related β -cyclase adds a ring at both ends. The DNA of the present invention is shown in Figure 4 and SEQ ID NO: 1. A plasmid containing this gene was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville MD 20852 on March 4, 1996 under ATCC accession number 98005 (pATeps - *A. thaliana*).

The present inventors have also isolated the gene encoding the enzyme, β -carotene hydroxylase, which is responsible for hydroxylating the β endgroup in carotenoids. The DNA of the present invention is shown in SEQ ID NO: 3 and Figure 5. The full length gene product hydroxylates both end groups of β -carotene as do products of genes which encode proteins truncated by up to 50 amino acids from the N-terminus. Products of genes which encode proteins truncated between about 60-110 amino acids from the N-terminus preferentially hydroxylates only one ring. A plasmid containing this gene was deposited with the American Type

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Culture Collection, 12301 Parklawn Drive, Rockville MD 20852 on March 4, 1996 under ATCC accession number 98003 (pATOHB - *A. thaliana*).

Eukaryotic genes which encode enzymes which produce novel or rare carotenoids

The present invention also relates to novel enzymes which can transform known carotenoids into novel or rare products. That is, currently ϵ -carotene (see figure 2) and γ -carotene can only be isolated in minor amounts. As described below, an enzyme can be produced which would transform lycopene to γ -carotene and lycopene to ϵ -carotene. With these products in hand, bulk synthesis of other carotenoids derived from them are possible. For example, ϵ -carotene can be hydroxylated to form an isomer of lutein (1 ϵ - and 1 β -ring) and zeaxanthin (2 β -rings) where both endgroups are, instead, ϵ -rings.

The eukaryotic genes in the carotenoid biosynthetic pathway differ from their prokaryotic counterparts in their 5' region. As used herein, the 5' region is the region of eukaryotic DNA which precedes the initiation codon of the counterpart gene in prokaryotic DNA. That is, when the consensus areas of eukaryotic and prokaryotic genes are aligned, the eukaryotic genes contain additional coding sequences upstream of the prokaryotic initiation codon.

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The present inventors have found that the amount of the 5' region present can alter the activity of the eukaryotic enzyme. Instead of diminishing activity, truncating the 5' region of the eukaryotic gene results in an enzyme with a different specificity. Thus, the present invention relates to enzymes which are truncated to within 0-50, preferably 0-25, codons of the 5' initiation codon of their prokaryotic counterparts as determined by alignment maps.

For example, as discussed above, when the gene encoding *A. thaliana* β -carotene hydroxylase was truncated, the resulting enzyme catalyzed the formation of β -cryptoxanthin as major product and zeaxanthin as minor product; in contrast to its normal production of zeaxanthin.

In addition to novel enzymes produced by truncating the 5' region of known enzymes, novel enzymes which can participate in the formation of novel carotenoids can be formed by replacing portions of one gene with an analogous sequence from a structurally related gene. For example, β -cyclase and ϵ -cyclase are structurally related (see Figure 13). By replacing a portion of β -lycopene cyclase with the analogous portion of ϵ -cyclase, an enzyme which produces γ -carotene will be produced (1 endgroup). Further, by replacing a portion of the ϵ -lycopene cyclase with the analogous portion of β -cyclase, an enzyme which produces ϵ -carotene will be produced (ϵ -cyclase normally produces a compound with 1 ϵ -endgroup (δ -carotene) not 2). Similarly, β -hydroxylase could

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be modified to produce enzymes of novel function by creation of hybrids with ϵ -hydroxylase.

Vectors

The genes encoding the carotenoid enzymes as described above, when cloned into a suitable expression vector, can be used to overexpress these enzymes in a plant expression system or to inhibit the expression of these enzymes. For example, a vector containing the gene encoding ϵ -cyclase can be used to increase the amount of α -carotene in an organism and thereby alter the nutritional value, pharmacology and visual appearance value of the organism.

In a preferred embodiment, the vectors of the present invention contain a DNA encoding an eukaryotic IPP isomerase upstream of a DNA encoding a second eukaryotic carotenoid enzyme. The inventors have discovered that inclusion of an IPP isomerase gene increases the supply of substrate for the carotenoid pathway; thereby enhancing the production of carotenoid endproducts. This is apparent from the much deeper pigmentation in carotenoid-accumulating colonies of *E. coli* which also contain one of the aforementioned IPP isomerase genes when compared to colonies that lack this additional IPP isomerase gene. Similarly, a vector comprising an IPP isomerase gene can be used to enhance production of any secondary metabolite of dimethylallyl pyrophosphate (such as isoprenoids, steroids, carotenoids, etc.).

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Alternatively, an anti-sense strand of one of the above genes can be inserted into a vector. For example, the ϵ -cyclase gene can be inserted into a vector and incorporated into the genomic DNA of a host, thereby inhibiting the synthesis of ϵ, β carotenoids (lutein and α -carotene) and enhancing the synthesis of β, β carotenoids (zeaxanthin and β -carotene).

Suitable vectors according to the present invention comprise a eukaryotic gene encoding an enzyme involved in carotenoid biosynthesis or metabolism and a suitable promoter for the host can be constructed using techniques well known in the art (for example Sambrook et al., Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

Suitable vectors for eukaryotic expression in plants are described in Frey et al., Plant J. (1995) 8(5):693 and Misawa et al, 1994a; incorporated herein by reference.

Suitable vectors for prokaryotic expression include pACYC184, pUC119, and pBR322 (available from New England BioLabs, Beverly, MA) and pTreHis (Invitrogen) and pET28 (Novagene) and derivatives thereof.

The vectors of the present invention can additionally contain regulatory elements such as promoters, repressors selectable markers such as antibiotic resistance genes, etc.

Hosts

Host systems according to the present invention can comprise any organism that already produces carotenoids or which has been genetically modified to produce carotenoids. The IPP isomerase genes are more broadly applicable for enhancing production of any product dependent on DMAPP as a precursor.

Organisms which already produce carotenoids include plants, algae, some yeasts, fungi and cyanobacteria and other photosynthetic bacteria. Transformation of these hosts with vectors according to the present invention can be done using standard techniques such as those described in Misawa et al., (1990) supra; Hundle et al., (1993) supra; Hundle et al., (1991) supra; Misawa et al., (1991) supra; Sandmann et al., supra; and Schnurr et al., supra; all incorporated herein by reference.

Alternatively, transgenic organisms can be constructed which include the DNA sequences of the present invention (Bird et al, 1991; Bramley et al, 1992; Misawa et al, 1994a; Misawa et al, 1994b; Cunningham et al, 1993). The incorporation of these sequences can allow the controlling of carotenoid biosynthesis, content, or composition in the host cell. These transgenic systems can be constructed to incorporate sequences which allow over-expression of the carotenoid genes of the present invention. Transgenic systems can also be constructed containing antisense expression of the DNA sequences of the

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present invention. Such antisense expression would result in the accumulation of the substrates of the substrates of the enzyme encoded by the sense strand.

A method for screening for eukaryotic genes which encode enzymes involved in carotenoid biosynthesis

The method of the present invention comprises transforming a prokaryotic host with a DNA which may contain a eukaryotic or prokaryotic carotenoid biosynthetic gene; culturing said transformed host to obtain colonies; and screening for colonies exhibiting a different color than colonies of the untransformed host.

Suitable hosts include *E. coli*, cyanobacteria such as *Synechococcus* and *Synechocystis*, alga and plant cells. *E. coli* are preferred.

In a preferred embodiment, the above "color complementation test" can be enhanced by using mutants which are either (1) deficient in at least one carotenoid biosynthetic gene or (2) overexpress at least one carotenoid biosynthetic gene. In either case, such mutants will accumulate carotenoid precursors.

Prokaryotic and eukaryotic DNA libraries can be screened in total for the presence of genes of carotenoid biosynthesis, metabolism and degradation. Preferred organisms to be screened include photosynthetic organisms.

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E. coli can be transformed with these eukaryotic cDNA libraries using conventional methods such as those described in Sambrook et al, 1989 and according to protocols described by the venders of the cloning vectors.

For example, the cDNA libraries in bacteriophage vectors such as lambdaZAP (Stratagene) or lambdaZIPLOX (Gibco BRL) can be excised en masse and used to transform *E. coli* can be inserted into suitable vectors and these vectors can be used to transform *E. coli*. Suitable vectors include pACYC184, pUC119, pBR322 (available from New England BioLabs, Beverly, MA). pACYC is preferred.

Transformed *E. coli* can be cultured using conventional techniques. The culture broth preferably contains antibiotics to select and maintain plasmids. Suitable antibiotics include penicillin, ampicillin, chloramphenicol, etc. Culturing is typically conducted at 20-40°C, preferably at room temperature (20-25°C), for 12 hours to 7 days.

Cultures are plated and the plates are screened visually for colonies with a different color than the colonies of the untransformed host *E. coli*. For example, *E. coli* transformed with the plasmid, pAC-BETA (described below), produce yellow colonies that accumulate β -carotene. After transformation with a cDNA library, colonies which contain a different hue than those formed by *E. coli*/pAC-BETA would be expected to contain enzymes which modify the structure or degree of expression of β -carotene. Similar standards can be engineered

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which overexpress earlier products in carotenoid biosynthesis, such as lycopene, γ -carotene, etc.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

EXAMPLE

I. Isolation of β -carotene hydroxylase

Plasmid Construction

An 8.6kb BglII fragment containing the carotenoid biosynthetic genes of *Erwinia herbicola* was first cloned in the BamHI site of plasmid vector pACYC184 (chloramphenicol resistant), and then a 1.1kb BamHI fragment containing the β -carotene hydroxylase (*CrtZ*) was deleted. The resulting plasmid, pAC-BETA, contains all the genes for the formation of β -carotene. *E.coli* strains containing this plasmid accumulate β -carotene and form yellow colonies (Cunningham et al., 1994).

A full length gene encoding IPP isomerase of *Haematococcus pluvialis* (HP04) was first cut out with BamHI-KpnI from pBluescript SK+, and then cloned into a pTrcHisA vector with high-level expression from the trc promoter (Invitrogen Inc.). A fragment containing the IPP isomerase and trc promoter was excised with EcoRV-KpnI and cloned in

HindIII site of pAC-BETA. *E.coli* cells transformed with this new plasmid pAC-BETA-04 form orange (deep yellow) colonies on LB plates and accumulate more β -carotene than cells that contain pAC-BETA.

Screening of the Arabidopsis cDNA Library

Several λ cDNA expression libraries of *Arabidopsis* were obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH) (Kieber et al., 1993). The λ cDNA libraries were excised *in vivo* using Stratagene's ExAssist SOLR system to produce a phagemid cDNA library wherein each clone also contained an ampicillin.

E.coli strain DH10BZIP was chosen as the host cells for the screening and pigment production. DH10B cells were transformed with plasmid pAC-BETA-04 and were plated on LB agar plates containing chloramphenicol at 50 μ g/ml (from United States Biochemical Corporation). The phagemid *Arabidopsis* cDNA library was then introduced into DH10B cells already containing pAC-BETA-04. Transformed cells containing both pAC-BETA-04 and *Arabidopsis* cDNA were selected on chloramphenicol plus ampicillin (150 μ g/ml) agar plates. Maximum color development occurred after 5 days incubation at room temperature, and lighter yellow colonies were selected. Selected colonies were inoculated into 3 ml liquid LB medium containing ampicillin and chloramphenicol, and cultures were incubated. Cells were then pelleted and extracted in 80 μ l

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100% acetone in microfuge tubes. After centrifugation, pigmented supernatant was spotted on silica gel thin-layer chromatography (TLC) plates, and developed with a hexane; ether (1:1) solvent system. β -carotene hydroxylase clones were identified based on the appearance of zeaxanthin on TLC plate.

Subcloning and Sequencing

The β -carotene hydroxylase cDNA was isolated by standard procedures (Sambrook et al., 1989). Restriction maps showed that three independent inserts (1.9kb, 0.9kb and 0.8kb) existed in the cDNA. To determine which cDNA insert confers the β -carotene hydroxylase activity, plasmid DNA was digested with NotI (a site in the adaptor of the cDNA library) and three inserts were subcloned into NotI site of SK vectors. These subclones were used to transform *E. coli* cells containing pAC-BETA-04 again to test the hydroxylase activity. A fragment of 0.95kb, later shown to contain the hydroxylase gene, was also blunt-ended and cloned into pTrcHis A,B,C vectors. To remove the N terminal sequence, a restriction site (BglII) was used that lies just before the conserved sequence with bacterial genes. A BglII-XhoI fragment was directionally cloned in BamHI-XhoI digested trc vectors. Functional clones were identified by the color complementation test. A β -carotene hydroxylase enzyme produces a colony with

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a lighter yellow color than is found in cells containing PAC-BETA-04 alone.

Arabidopsis β -carotene hydroxylase was sequenced completely on both strands on an automatic sequencer (Applied Biosystems, Model 373A, Version 2.0.1S).

Pigment Analysis

A single colony was used to inoculate 50 ml of LB containing ampicillin and chloramphenicol in a 250-ml flask. Cultures were incubated at 28°C for 36 hours with gentle shaking, and then harvested at 5000 rpm in an SS-34 rotor. The cells were washed once with distilled H₂O and resuspended with 0.5 ml of water. The extraction procedures and HPLC were essentially as described previously (Cunningham et al, 1994).

II. Isolation of ϵ cyclase

Plasmid Construction

Construction of plasmids PAC-LYC, PAC-NEUR, and PAC-ZETA is described in Cunningham et al., (1994). In brief, the appropriate carotenoid biosynthetic genes from *Erwinia herbicola*, *Rhodobacter capsulatus*, and *Synechococcus* sp. strain PCC7942 were cloned in the plasmid vector pACYC184 (New England BioLabs, Beverly, MA). Cultures of *E. coli* containing the plasmids PAC-ZETA, PAC-NEUR, and PAC-LYC, accumulate β -carotene, neurosporene, and lycopene, respectively. The plasmid PAC-ZETA was constructed as follows: an 8.6-kb BglII

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fragment containing the carotenoid biosynthetic genes of *E. herbicola* (GenBank M87280; Hundle et al., 1991) was obtained after partial digestion of plasmid pPL376 (Perry et al., 1986; Tuveson et al., 1986) and cloned in the BamHI site of pACYC184 to give the plasmid pAC-EHER. Deletion of adjacent 0.8- and 1.1-kb BamHI-BamHI fragments (deletion Z in Cunningham et al., 1994), and of a 1.1 kb SalI-SalI fragment (deletion X) served to remove most of the coding regions for the *E. herbicola* β -carotene hydroxylase (crt gene) and zeaxanthin glucosyltransferase (crtX gene), respectively. The resulting plasmid, pAC-BETA, retains functional genes for geranylgeranyl pyrophosphate synthase (crtE), phytoene synthase (crtB), phytoene desaturase (crtI), and lycopene cyclase (crtY). Cells of *E. coli* containing this plasmid form yellow colonies and accumulate β -carotene. A plasmid containing both the ϵ - and β -cyclase cDNAs of *A. thaliana* was constructed by excising the ϵ cyclase in clone y2 as a PvuI-PvuII fragment and ligating this piece in the SnaBI site of a plasmid (pSPORT 1 from GIBCO-BRL) that already contained the β cyclase.

Organisms and Growth Conditions

E. coli strains TOP10 and TOP10 F' (obtained from Invitrogen Corporation, San Diego, CA) and XL1-Blue (Stratagene) were grown in Luria-Bertani (LB) medium (Sambrook et al., 1989) at 37°C in darkness on a platform shaker at 225

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cycles per min. Media components were from Difco (yeast extract and tryptone) or Sigma (NaCl). Ampicillin at 150 $\mu\text{g/mL}$ and/or chloramphenicol at 50 $\mu\text{g/mL}$ (both from United States Biochemical Corporation) were used, as appropriate, for selection and maintenance of plasmids.

Mass Excision and Color Complementation Screening of an *A. thaliana* cDNA Library

A size-fractionated 1-2 kB cDNA library of *A. thaliana* in lambda ZAPII (Kieber et al., 1993) was obtained from the Arabidopsis Biological Resource Center at The Ohio State University (stock number CD4-14). Other size fractionated libraries were also obtained (stock numbers CD4-13, CD4-15, and CD4-16). An aliquot of each library was treated to cause a mass excision of the cDNAs and thereby produce a phagemid library according to the instructions provided by the supplier of the cloning vector (Stratagene; *E. coli* strain XL1-Blue and the helper phage R408 were used). The titre of the excised phagemid was determined and the library was introduced into a lycopene-accumulating strain of *E. coli* TOP10 F' (this strain contained the plasmid pAC-LYC) by incubation of the phagemid with the *E. coli* cells for 15 min at 37°C. Cells had been grown overnight at 30°C in LB medium supplemented with 2% (w/v) maltose and 10 mM MgSO_4 (final concentration), and harvested in 1.5 ml microfuge tubes at a setting of 3 on an Eppendorf microfuge (5415C) for 10 min. The pellets were

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resuspended in 10 mM MgSO₄ to a volume equal to one-half that of the initial culture volume. Transformants were spread on large (150 mm diameter) LB agar petri plates containing antibiotics to provide for selection of cDNA clones (ampicillin) and maintenance of pAC-LYC (chloramphenicol). Approximately 10,000 colony forming units were spread on each plate. Petri plates were incubated at 37°C for 16 hr and then at room temperature for 2 to 7 days to allow maximum color development. Plates were screened visually with the aid of an illuminated 3x magnifier and a low power stage-dissecting microscope for the rare, pale pinkish-yellow to deep-yellow colonies that could be observed in the background of pink colonies. A colony color of yellow or pinkish-yellow was taken as presumptive evidence of a cyclization activity. These yellow colonies were collected with sterile toothpicks and used to inoculate 3ml of LB medium in culture tubes with overnight growth at 37°C and shaking at 225 cycles/min. Cultures were split into two aliquots in microfuge tubes and harvested by centrifugation at a setting of 5 in an Eppendorf 5415C microfuge. After discarding the liquid, one pellet was frozen for later purification of plasmid DNA. To the second pellet was added 1.5 ml EtOH, and the pellet was resuspended by vortex mixing, and extraction was allowed to proceed in the dark for 15-30 min with occasional remixing. Insoluble materials were pelleted by centrifugation at maximum speed for 10 min in a microfuge. Absorption spectra of the supernatant

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fluids were recorded from 350-550 nm with a Perkin Elmer lambda six spectrophotometer.

Analysis of isolated clones

Eight of the yellow colonies contained β -carotene indicating that a single gene product catalyzes both cyclizations required to form the two β endgroups of the symmetrical β -carotene from the symmetrical precursor lycopene. One of the yellow colonies contained a pigment with the spectrum characteristic of δ -carotene, a monocyclic carotenoid with a single ϵ endgroup. Unlike the β cyclase, this ϵ cyclase appears unable to carry out a second cyclization at the other end of the molecule.

The observation that ϵ cyclase is unable to form two cyclic ϵ endgroups (e.g. the bicyclic ϵ -carotene) illuminates the mechanism by which plants can coordinate and control the flow of substrate into carotenoids derived from β -carotene versus those derived from α -carotene and also can prevent the formation of carotenoids with two ϵ endgroups.

The availability of the *A. thaliana* gene encoding the ϵ cyclase enables the directed manipulation of plant and algal species for modification of carotenoid content and composition. Through inactivation of the ϵ cyclase, whether at the gene level by deletion of the gene or by insertional inactivation or by reduction of the amount of enzyme formed (by such as antisense technology), one may increase the

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formation of β -carotene and other pigments derived from it. Since vitamin A is derived only from carotenoids with β endgroups, an enhancement of the production of β -carotene versus α -carotene may enhance nutritional value of crop plants. Reduction of carotenoids with ϵ endgroups may also be of value in modifying the color properties of crop plants and specific tissues of these plants. Alternatively, where production of α -carotene, or pigments such as lutein that are derived from α -carotene, is desirable, whether for the color properties, nutritional value or other reason, one may overexpress the ϵ cyclase or express it in specific tissues. Wherever agronomic value of a crop is related to pigmentation provided by carotenoid pigments the directed manipulation of expression of the ϵ cyclase gene and/or production of the enzyme may be of commercial value.

The predicted amino acid sequence of the *A. thaliana* ϵ cyclase enzyme was determined. A comparison of the amino acid sequences of the β and ϵ cyclase enzymes of *Arabidopsis thaliana* (Fig. 13) as predicted by the DNA sequence of the respective genes (Fig. 4 for the ϵ cyclase cDNA sequence), indicates that these two enzymes have many regions of sequence similarity, but they are only about 37% identical overall at the amino acid level. The degree of sequence identity at the DNA base level, only about 50%, is sufficiently low such that

-27-

we and others have been unable to detect this gene by hybridization using the β cyclase as a probe in DNA gel blot experiments.

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Having now fully described the invention, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the invention as set forth herein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: CUNNINGHAM JR., FRANCIS X.
SUN, ZAIREN
- (ii) TITLE OF INVENTION: GENES OF CAROTENOID BIOSYNTHESIS AND
METABOLISM AND A SYSTEM FOR SCREENING SUCH GENES
- (iii) NUMBER OF SEQUENCES: 21
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: OBLON, SPIVAK, MCCLELLAND, MAIER & NEUSTADT,
P.C.
 - (B) STREET: 1755 S. JEFFERSON DAVIS HIGHWAY, SUITE 400
 - (C) CITY: ARLINGTON
 - (D) STATE: VA
 - (E) COUNTRY: USA
 - (F) ZIP: 22202
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/624,125
 - (B) FILING DATE: 29-MAR-1996
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: KELBER, STEVEN B.
 - (B) REGISTRATION NUMBER: 30,073
 - (C) REFERENCE/DOCKET NUMBER: 2747-063-27
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 703-413-3000
 - (B) TELEFAX: 703-413-2220

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1860 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 109..1680
 - (D) OTHER INFORMATION: /product= "E-CYCLASE FROM A.
THALIANA"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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| Met Glu Cys | |
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| GTT GGG GCT AGG AAT TTC GCA GCA ATG GCG GTT TCA ACA TTT CCG TCA | 165 |
| Val Gly Ala Arg Asn Phe Ala Ala Met Ala Val Ser Thr Phe Pro Ser | |
| 5 10 15 | |
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| Trp Ser Cys Arg Arg Lys Phe Pro Val Val Lys Arg Tyr Ser Tyr Arg | |
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| Asn Ile Arg Phe Gly Leu Cys Ser Val Arg Ala Ser Gly Gly Gly Ser | |
| 40 45 50 | |
| TCC GGT AGT GAG AGT TGT GTA GCG GTG AGA GAA GAT TTC GCT GAC GAA | 309 |
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| 55 60 65 | |
| GAA GAT TTT GTG AAA GCT GGT GGT TCT GAG ATT CTA TTT GTT CAA ATG | 357 |
| Glu Asp Phe Val Lys Ala Gly Gly Ser Glu Ile Leu Phe Val Gln Met | |
| 70 75 80 | |
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| Gln Gln Asn Lys Asp Met Asp Glu Gln Ser Lys Leu Val Asp Lys Leu | |
| 85 90 95 | |
| CCT CCT ATA TCA ATT GGT GAT GGT GCT TTG GAT CAT GTG GTT ATT GGT | 453 |
| Pro Pro Ile Ser Ile Gly Asp Gly Ala Leu Asp His Val Val Ile Gly | |
| 100 105 110 115 | |
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| Cys Gly Pro Ala Gly Leu Ala Leu Ala Ala Glu Ser Ala Lys Leu Gly | |
| 120 125 130 | |
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| Gly Val Trp Glu Asp Glu Phe Asn Asp Leu Gly Leu Gln Lys Cys Ile | |
| 150 155 160 | |
| GAG CAT GTT TGG AGA GAG ACT ATT GTG TAT CTG GAT GAT GAC AAG CCT | 645 |
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| 165 170 175 | |
| ATT ACC ATT GGC CGT GCT TAT GGA AGA GTT AGT CGA CGT TTG CTC CAT | 693 |
| Ile Thr Ile Gly Arg Ala Tyr Gly Arg Val Ser Arg Arg Leu Leu His | |
| 180 185 190 195 | |

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| Lys Arg Gln Arg Ala Phe Phe Leu Phe Gly Leu Ala Leu Ile Val Gln | | |
| 440 | 445 | 450 |
| TTC GAT ACC GAA GGC ATT AGA AGC TTC TTC CGT ACT TTC TTC CGC CTT | | 1509 |
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| Pro Lys Trp Met Trp Gln Gly Phe Leu Gly Ser Thr Leu Thr Ser Gly | | |
| 470 | 475 | 480 |
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| 520 | | |
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 524 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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| Phe | Pro | Ser | Trp | Ser | Cys | Arg | Arg | Lys | Phe | Pro | Val | Val | Lys | Arg | Tyr |
| | | | 20 | | | | | 25 | | | | | 30 | | |
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| | | 35 | | | | | 40 | | | | | 45 | | | |
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| | 50 | | | | | 55 | | | | | 60 | | | | |

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 115 120 125
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 130 135 140
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 Lys Cys Ile Glu His Val Trp Arg Glu Thr Ile Val Tyr Leu Asp Asp
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 Thr Phe Leu Tyr Ala Met Pro Met Thr Lys Ser Arg Leu Phe Phe Glu
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37

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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 956 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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| GCTCTTTCTC CTCCTCCTCT ACCGATTTC GACTCCGCCT CCCGAAATCC TTATCCGGAT | 60 |
| TCTCTCCGTC TCTTCGATTT AAACGCTTTT CTGTCTGTTA CGTCGTCGAA GAACGGAGAC | 120 |
| AGAATTCTCC GATTGAGAAC GATGAGAGAC CGGAGAGCAC GAGCTCCACA AACGCTATAG | 180 |
| ACGCTGAGTA TCTGGCGTTG CGTTTGGCGG AGAAATTGGA GAGGAAGAAA TCGGAGAGGT | 240 |
| CCACTTATCT AATCGCTGCT ATGTTGTCGA GCTTTGGTAT CACTTCTATG GCTGTTATGG | 300 |
| CTGTTTACTA CAGATTCTCT TGGCAAATGG AGGGAGGTGA GATCTCAATG TTGGAAATGT | 360 |


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TTGGTACATT TGCTCTCTCT GTTGGTGCTG CTGTTGGTAT GGAATTCTGG GCAAGATGGG      420
CTCATAGAGC TCTGTGGCAC GCTTCTCTAT GGAATATGCA TGAGTCACAT CACAAACCAA      480
GAGAAGGACC GTTTGAGCTA AACGATGTTT TTGCTATAGT GAACGCTGGT CCAGCGATTG      540
GTCTCCTCTC TTATGGATTC TTCAATAAAG GACTCGTTCC TGGTCTCTGC TTTGGCGCCG      600
GGTTAGGCAT AACGGTGTTT GGAATCGCCT ACATGTTTGT CCACGATGGT CTCGTGCACA      660
AGCGTTTCCC TGTAGGTCCC ATCGCCGACG TCCCTTACCT CCGAAAGGTC GCCGCCGCTC      720
ACCAGCTACA TCACACAGAC AAGTTCAATG GTGTACCATA TGGACTGTTT CTTGGACCCA      780
AGGAATTGGA AGAAGTTGGA GGAAATGAAG AGTTAGATAA GGAGATTAGT CGGAGAATCA      840
AATCATACAA AAAGGCCTCG GGCTCCGGGT CGAGTTCGAG TTCTTGACTT TAAACAAGTT      900
TTAAATCCCA AATTCTTTTT TTGTCTTCTG TCATTATGAT CATCTTAAGA CGGTCT      956

```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 294 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Ser Phe Ser Ser Ser Ser Thr Asp Phe Arg Leu Arg Leu Pro Lys Ser
1           5           10           15
Leu Ser Gly Phe Ser Pro Ser Leu Arg Phe Lys Arg Phe Ser Val Cys
20           25           30
Tyr Val Val Glu Glu Arg Arg Gln Asn Ser Pro Ile Glu Asn Asp Glu
35           40           45
Arg Pro Glu Ser Thr Ser Ser Thr Asn Ala Ile Asp Ala Glu Tyr Leu
50           55           60
Ala Leu Arg Leu Ala Glu Lys Leu Glu Arg Lys Lys Ser Glu Arg Ser
65           70           75           80
Thr Tyr Leu Ile Ala Ala Met Leu Ser Ser Phe Gly Ile Thr Ser Met
85           90           95
Ala Val Met Ala Val Tyr Tyr Arg Phe Ser Trp Gln Met Glu Gly Gly
100          105          110
Glu Ile Ser Met Leu Glu Met Phe Gly Thr Phe Ala Leu Ser Val Gly

```

| | | | | |
|---|-----|-----|-----|-----|
| 115 | | 120 | | 125 |
| Ala Ala Val Gly Met Glu Phe Trp Ala Arg Trp Ala His Arg Ala Leu | | | | |
| 130 | | 135 | | 140 |
| Trp His Ala Ser Leu Trp Met Asn His Glu Ser His His Lys Pro Arg | | | | |
| 145 | | 150 | | 155 |
| Glu Gly Pro Phe Glu Leu Asn Asp Val Phe Ala Ile Val Asn Ala Gly | | | | |
| | 165 | | 170 | 175 |
| Pro Ala Ile Gly Leu Leu Ser Tyr Gly Phe Phe Asn Lys Gly Leu Val | | | | |
| | 180 | | 185 | 190 |
| Pro Gly Leu Cys Phe Gly Ala Gly Leu Gly Ile Thr Val Phe Gly Ile | | | | |
| | 195 | | 200 | 205 |
| Ala Tyr Met Phe Val His Asp Gly Leu Val His Lys Arg Phe Pro Val | | | | |
| | 210 | | 215 | 220 |
| Gly Pro Ile Ala Asp Val Pro Tyr Leu Arg Lys Val Ala Ala Ala His | | | | |
| 225 | | 230 | | 235 |
| Gln Leu His His Thr Asp Lys Phe Asn Gly Val Pro Tyr Gly Leu Phe | | | | |
| | 245 | | 250 | 255 |
| Leu Gly Pro Lys Glu Leu Glu Glu Val Gly Gly Asn Glu Glu Leu Asp | | | | |
| | 260 | | 265 | 270 |
| Lys Glu Ile Ser Arg Arg Ile Lys Ser Tyr Lys Lys Ala Ser Gly Ser | | | | |
| | 275 | | 280 | 285 |
| Gly Ser Ser Ser Ser Ser | | | | |
| 290 | | | | |

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 162 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Thr Gln Phe Leu Ile Val Val Ala Thr Val Leu Val Met Glu Leu
1 5 10 15
Thr Ala Tyr Ser Val His Arg Trp Ile Met His Gly Pro Leu Gly Trp
20 25 30
Gly Trp His Lys Ser His His Glu Glu His Asp His Ala Leu Glu Lys

40

| | | |
|---|-----|-----|
| 35 | 40 | 45 |
| Asn Asp Leu Tyr Gly Val Val Phe Ala Val Leu Ala Thr Ile Leu Phe | | |
| 50 | 55 | 60 |
| Thr Val Gly Ala Tyr Trp Trp Pro Val Leu Trp Trp Ile Ala Leu Gly | | |
| 65 | 70 | 75 |
| Met Thr Val Tyr Gly Leu Ile Tyr Phe Ile Leu His Asp Gly Leu Val | | |
| 85 | 90 | 95 |
| His Gln Arg Trp Pro Phe Arg Tyr Ile Pro Arg Arg Gly Tyr Phe Arg | | |
| 100 | 105 | 110 |
| Arg Leu Tyr Gln Ala His Arg Leu His His Ala Val Glu Gly Arg Asp | | |
| 115 | 120 | 125 |
| His Cys Val Ser Phe Gly Phe Ile Tyr Ala Pro Pro Val Asp Lys Leu | | |
| 130 | 135 | 140 |
| Lys Gln Asp Leu Lys Arg Ser Gly Val Leu Arg Pro Gln Asp Glu Arg | | |
| 145 | 150 | 155 |
| | | 160 |
| Pro Ser | | |

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 175 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

| | | |
|---|----|----|
| Met Leu Asn Ser Leu Ile Val Ile Leu Ser Val Ile Ala Met Glu Gly | | |
| 1 | 5 | 10 |
| Ile Ala Ala Phe Thr His Arg Tyr Ile Met His Gly Trp Gly Trp Arg | | |
| 20 | 25 | 30 |
| Trp His Glu Ser His His Thr Pro Arg Lys Gly Val Phe Glu Leu Asn | | |
| 35 | 40 | 45 |
| Asp Leu Phe Ala Val Val Phe Ala Gly Val Ala Ile Ala Leu Ile Ala | | |
| 50 | 55 | 60 |
| Val Gly Thr Ala Gly Val Trp Pro Leu Gln Trp Ile Gly Cys Gly Met | | |
| 65 | 70 | 75 |
| Thr Val Tyr Gly Leu Leu Tyr Phe Leu Val His Asp Gly Leu Val His | | |

41

| | | |
|---|---------------------------------|-----|
| 85 | 90 | 95 |
| Gln Arg Trp Pro Phe His Trp Ile | Pro Arg Arg Gly Tyr Leu Lys Arg | |
| 100 | 105 | 110 |
| Leu Tyr Val Ala His Arg Leu His His | Ala Val Arg Gly Arg Glu Gly | |
| 115 | 120 | 125 |
| Cys Val Ser Phe Gly Phe Ile Tyr Ala Arg Lys | Pro Ala Asp Leu Gln | |
| 130 | 135 | 140 |
| Ala Ile Leu Arg Glu Arg His Gly Arg Pro Pro Lys Arg Asp Ala Ala | | |
| 145 | 150 | 155 |
| Lys Asp Arg Pro Asp Ala Ala Ser Pro Ser Ser Ser Ser Pro Glu | | |
| 165 | 170 | 175 |

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 175 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

| | |
|---|-----|
| Met Leu Trp Ile Trp Asn Ala Leu Ile Val Phe Val Thr Val Ile Gly | |
| 1 | 15 |
| Met Glu Val Ile Ala Ala Leu Ala His Lys Tyr Ile Met His Gly Trp | |
| 20 | 30 |
| Gly Trp Gly Trp His Leu Ser His His Glu Pro Arg Lys Gly Ala Phe | |
| 35 | 45 |
| Glu Val Asn Asp Leu Tyr Ala Val Val Phe Ala Ala Leu Ser Ile Leu | |
| 50 | 60 |
| Leu Ile Tyr Leu Gly Ser Thr Gly Met Trp Pro Leu Gln Trp Ile Gly | |
| 65 | 80 |
| Ala Gly Met Thr Ala Tyr Gly Leu Leu Tyr Phe Met Val His Asp Gly | |
| 85 | 95 |
| Leu Val His Gln Arg Trp Pro Phe Arg Tyr Ile Pro Arg Lys Gly Tyr | |
| 100 | 110 |
| Leu Lys Arg Leu Tyr Met Ala His Arg Met His His Ala Val Arg Gly | |
| 115 | 125 |
| Lys Glu Gly Cys Val Ser Phe Glv Phe Leu Tvr Ala Pro Pro Leu Ser | |

| | | | |
|---|-----|-----|-----|
| 130 | 135 | 140 | |
| Lys Leu Gln Ala Thr Leu Arg Glu Arg His Gly Ala Arg Ala Gly Ala | | | |
| 145 | 150 | 155 | 160 |
| Ala Arg Asp Ala Gln Gly Gly Glu Asp Glu Pro Ala Ser Gly Lys | | | |
| 165 | 170 | 175 | |

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 162 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

| | | | |
|---|-----|-----|-----|
| Met Thr Asn Phe Leu Ile Val Val Ala Thr Val Leu Val Met Glu Leu | | | |
| 1 | 5 | 10 | 15 |
| Thr Ala Tyr Ser Val His Arg Trp Ile Met His Gly Pro Leu Gly Trp | | | |
| 20 | 25 | 30 | |
| Gly Trp His Lys Ser His His Glu Glu His Asp His Ala Leu Glu Lys | | | |
| 35 | 40 | 45 | |
| Asn Asp Leu Tyr Gly Leu Val Phe Ala Val Ile Ala Thr Val Leu Phe | | | |
| 50 | 55 | 60 | |
| Thr Val Gly Trp Ile Trp Ala Pro Val Leu Trp Trp Ile Ala Leu Gly | | | |
| 65 | 70 | 75 | 80 |
| Met Thr Val Tyr Gly Leu Ile Tyr Phe Val Leu His Asp Gly Leu Val | | | |
| 85 | 90 | 95 | |
| His Trp Arg Trp Pro Phe Arg Tyr Ile Pro Arg Lys Gly Tyr Ala Arg | | | |
| 100 | 105 | 110 | |
| Arg Leu Tyr Gln Ala His Arg Leu His His Ala Val Glu Gly Arg Asp | | | |
| 115 | 120 | 125 | |
| His Cys Val Ser Phe Gly Phe Ile Tyr Ala Pro Pro Val Asp Lys Leu | | | |
| 130 | 135 | 140 | |
| Lys Gln Asp Leu Lys Met Ser Gly Val Leu Arg Ala Glu Ala Gln Glu | | | |
| 145 | 150 | 155 | 160 |
| Arg Thr | | | |

(2) INFORMATION FOR SEQ ID NO:9:

SUBSTITUTE SHEET (RULE 26)

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 954 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

| | |
|--|-----|
| CCACGGGTCC GCCTCCCCGT TTTTTCCTGA TCCGATCTCC GGTGCCGAGG ACTCAGCTGT | 60 |
| TTGTTGCGCG TTTCTCAGCC GTCACCATGA CCGATTCTAA CGATGCTGGA ATGGATGCTG | 120 |
| TTCAGAGACG ACTCATGTTT GAAGACGAAT GCATTCTCGT TGATGAAAAT AATCGTGTGG | 180 |
| TGGGACATGA CACTAAGTAT AACTGTCATC TGATGGAAAA GATTGAAGCT GAGAATTTAC | 240 |
| TTCACAGAGC TTTCACTGTG TTTTATTCA ACTCCAAGTA TGAGTTGCTT CTCCAGCAAC | 300 |
| GGTCAAAAAC AAAGGTTACT TTCCCACTTG TGTGGACAAA CACTTGTTGC AGCCATCCTC | 360 |
| TTTACCGTGA ATCCGAGCTT ATTGAAGAGA ATGTGCTTGG TGTAAGAAAT GCCGCACAAA | 420 |
| GGAAGCTTTT CGATGAGCTC GGTATTGTAG CAGAAGATGT ACCAGTCGAT GAGTTCACCTC | 480 |
| CCTTGGGACG CATGCTTTAC AAGGCACCTT CTGATGGGAA ATGGGGAGAG CACGAAGTTG | 540 |
| ACTATCTACT CTTCATCGTG CGGGATGTGA AGCTTCAACC AAACCCAGAT GAAGTGGCTG | 600 |
| AGATCAAGTA CGTGAGCAGG GAAGAGCTTA AGGAGCTGGT GAAGAAAGCA GATGCTGGCG | 660 |
| ATGAAGCTGT GAAACTATCT CCATGGTTCA GATTGGTGGT GGATAATTTC TTGATGAAGT | 720 |
| GGTGGGATCA TGTTGAGAAA GGAATATCA CTGAAGCTGC AGACATGAAA ACCATTACAC | 780 |
| AGCTCTGAAC TTTCCATAAG TTTTGGATCT TCCCCTTCCC ATAATAAAAT TAAGAGATGA | 840 |
| GACTTTTATT GATTACAGAC AAAACTGGCA ACAAATCTA TTCCTAGGAT TTTTTTTTGC | 900 |
| TTTTTATTTA CTTTGTATTC ATCTCTAGTT TAGTTTTCAT CTTAAAAAAA AAAA | 954 |

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 996 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

SUBSTITUTE SHEET (RULE 26)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

| | |
|--|-----|
| CACCAATGTC TGTTTCTTCT TTATTTAATC TCCCATTGAT TCGCCTCAGA TCTCTCGCTC | 60 |
| TTTCGTCTTC TTTTCTTCT TTCCGATTG CCCATCGTCC TCTGTCATCG ATTTCACCGA | 120 |
| GAAAGTTACC GAATTTTCGT GCTTTCTCTG GTACCGCTAT GACAGATACT AAAGATGCTG | 180 |
| GTATGGATGC TGTTTCAGAGA CGTCTCATGT TTGAGGATGA ATGCATTCTT GTTGATGAAA | 240 |
| CTGATCGTGT TGTGGGGCAT GTCAGCAAGT ATAATTGTCA TCTGATGGAA AATATTGAAG | 300 |
| CCAAGAATTT GCTGCACAGG GCTTTTAGTG TATTTTTATT CAACTCGAAG TATGAGTTGC | 360 |
| TTCTCCAGCA AAGGTCAAAC ACAAAGGTTA CGTTCCCTCT AGTGTGGACT AACACTTGTT | 420 |
| GCAGCCATCC TCTTTACCGT GAATCAGAGC TTATCCAGGA CAATGCACTA GGTGTGAGGA | 480 |
| ATGCTGCACA AAGAAAGCTT CTCGATGAGC TTGGTATTGT AGCTGAAGAT GTACCAGTCG | 540 |
| ATGAGTTCAC TCCCTTGGGA CGTATGCTGT ACAAGGCTCC TTCTGATGGC AAATGGGGAG | 600 |
| AGCATGAACT TGATTACTTG CTCTTCATCG TCGGAGACGT GAAGGTTCAA CCAAACCCAG | 660 |
| ATGAAGTAGC TGAGATCAAG TATGTGAGCC GGAAGAGCT GAAGGAGCTG GTGAAGAAAG | 720 |
| CAGATGCAGG TGAGGAAGGT TTGAACTGT CACCATGGTT CAGATTGGTG GTGGACAATT | 780 |
| TCTTGATGAA GTGGTGGGAT CATGTTGAGA AAGGAACTTT GGTGAAGCT ATAGACATGA | 840 |
| AAACCATCCA CAACTCTGA ACATCTTTTT TTAAAGTTTT TAAATCAATC AACTTTCTCT | 900 |
| TCATCATTTT TATCTTTTCG ATGATAATAA TTTGGGATAT GTGAGACACT TACAAAACCT | 960 |
| CCAAGCACCT CAGGCAATAA TAAAGTTTGC GGCCGC | 996 |

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1165 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

| | |
|---|-----|
| CTCGGTAGCT GGCCACAATC GCTATTTGGA ACCTGGCCCG GCGGCAGTCC GATGCCGCGA | 60 |
| TGCTTCGTTC GTTGCTCAGA GGCCTCACGC ATATCCCCCG CGTGAAGTCC GCCCAGCAGC | 120 |
| CCAGCTGTGC ACACGCGCGA CTCCAGTTTA AGCTCAGGAG CATGCAGATG ACGTCTATGC | 180 |

| | |
|---|------|
| AGCCCAGCAT CTCAGCCAAT CTGTCGCGCG CCGAGGACCG CACAGACCAC ATGAGGGGTG | 240 |
| CAAGCACCTG GGCAGGCGGG CAGTCGCAGG ATGAGCTGAT GCTGAAGGAC GAGTGCATCT | 300 |
| TGGTGGATGT TGAGGACAAC ATCACAGGCC ATGCCAGCAA GCTGGAGTGT CACAAGTTCC | 360 |
| TACCACATCA GCCTGCAGGC CTGCTGCACC GGGCCTTCTC TGTGTTCTCG TTTGACGATC | 420 |
| AGGGGCGACT GCTGCTGCAA CAGCGTGCAC GCTCAAAAAT CACCTTCCCA AGTGTGTGGA | 480 |
| CGAACACCTG CTGCAGCCAC CCTTTACATG GGCAGACCCC AGATGAGGTG GACCAACTAA | 540 |
| GCCAGGTGGC CGACGGAACA GTACCTGGCG CAAAGGCTGC TGCCATCCGC AAGTTGGAGC | 600 |
| ACGAGCTGGG GATACCAGCG CACCAGCTGC CGGCAAGCGC GTTTCGCTTC CTCACGCGTT | 660 |
| TGCACTACTG TGCCGCGGAC GTGCAGCCAG CTGCGACACA ATCAGCGCTC TGGGGCGAGC | 720 |
| ACGAAATGGA CTACATCTTG TTCATCCGGG CCAACGTCAC CTTGGCGCCC AACCTGACG | 780 |
| AGGTGGACGA AGTCAGGTAC GTGACGCAAG AGGAGCTGCG GCAGATGATG CAGCCGGACA | 840 |
| ACGGGCTGCA ATGGTCGCGG TGGTTTCGCA TCATCGCCGC GCGCTTCCTT GAGCGTTGGT | 900 |
| GGGCTGACCT GGACGCGGCC CTAAACACTG ACAAACACGA GGATTGGGGA ACGGTGCATC | 960 |
| ACATCAACGA AGCGTGAAAG CAGAAGCTGC AGGATGTGAA GACACGTCAT GGGGTGGAAT | 1020 |
| TGCGTACTTG GCAGCTTCGT ATCTCCTTTT TCTGAGACTG AACCTGCAGT CAGGTCCCAC | 1080 |
| AAGGTCAGGT AAAATGGCTC GATAAAATGT ACCGTCACCT TTTGTCGCGT ATACTGAACT | 1140 |
| CCAAGAGGTC AAAAAAAAAA AAAAA | 1165 |

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1135 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

| | |
|---|-----|
| CTCGGTAGCT GGCCACAATC GCTATTTGGA ACCTGGCCCG GCGGCAGTCC GATGCCGCGA | 60 |
| TGCTTCGTTT GTTGCTCAGA GGCCTCACGC ATATCCCGCG CGTGAACTCC GCCCAGCAGC | 120 |
| CCAGCTGTGC ACACGCGCGA CTCCAGTTTA AGCTCAGGAG CATGCAGCTG CTTTCCGAGG | 180 |
| ACCGCACAGA CCACATGAGG GGTGCAAGCA CCTGGGCGAG CGGGCAGTCG CAGGATGAGC | 240 |

| | |
|--|------|
| TGATGCTGAA GGACGAGTGC ATCTTGGTAG ATGTTGAGGA CAACATCACA GGCCATGCCA | 300 |
| GCAAGCTGGA GTGTCACAAG TTCCTACCAC ATCAGCCTGC AGGCCTGCTG CACCGGGCCT | 360 |
| TCTCTGTGTT CCTGTTTGAC GATCAGGGGC GACTGCTGCT GCAACAGCGT GCACGCTCAA | 420 |
| AAATCACCTT CCCAAGTGTG TGGACGAACA CTGCTGCAG CCACCCTTTA CATGGGCAGA | 480 |
| CCCCAGATGA GGTGGACCAA CTAAGCCAGG TGGCCGACGG AACAGTACCT GGCGCAAAGG | 540 |
| CTGCTGCCAT CCGCAAGTTG GAGCACGAGC TGGGGATACC AGCGCACCAG CTGCCGGCAA | 600 |
| GCGCGTTTCG CTTCTCAGC CGTTTGCACT ACTGTGCCGC GGACGTGCAG CCAGCTGCGA | 660 |
| CACAATCAGC GCTCTGGGGC GAGCACGAAA TGGACTACAT CTTGTTTCATC CGGGCCAACG | 720 |
| TCACCTTGGC GCCCAACCCT GACGAGGTGG ACGAAGTCAG GTACGTGACG CAAGAGGAGC | 780 |
| TGCGGCAGAT GATGCAGCCG GACAACGGGC TTCAATGGTC GCCGTGGTTT CGCATCATCG | 840 |
| CCGCGCGCTT CCTTGAGCGT TGGTGGGCTG ACCTGGACGC GGCCCTAAAC ACTGACAAAC | 900 |
| ACGAGGATTG GGGAACGGTG CATCACATCA ACGAAGCGTG AAGGCAGAAG CTGCAGGATG | 960 |
| TGAAGACACG TCATGGGGTG GAATTGCGTA CTTGGCAGCT TCGTATCTCC TTTTCTGAG | 1020 |
| ACTGAACCTG CAGAGCTAGA GTCAATGGTG CATCATATTC ATCGTCTCTC TTTTGTTTTA | 1080 |
| GACTAATCTG TAGCTAGAGT CACTGATGAA TCCTTTACAA CTTTCAAAAA AAAAA | 1135 |

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 960 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

| | |
|---|-----|
| CCAAAAACAA CTCAAATCTC CTCCGTCGCT CTTACTCCGC CATGGGTGAC GACTCCGGCA | 60 |
| TGGATGCTGT TCAGCGACGT CTCATGTTTG ACGATGAATG CATTTTGGTG GATGAGTGTG | 120 |
| ACAATGTGGT GGGACATGAT ACCAAATACA ATTGTCACTT GATGGAGAAG ATTGAAACAG | 180 |
| GTAAATGCT GCACAGAGCA TTCAGCGTTT TTCTATTCAA TTCAAAATAC GAGTTACTTC | 240 |
| TTCAGCAACG GTCTGCAACC AAGGTGACAT TTCCTTTAGT ATGGACCAAC ACCTGTTGCA | 300 |
| GCCATCCACT CTACAGAGAA TCCGAGCTTG TTCCCGAAAC GCCTGAGAGA ATGCTGCACA | 360 |

| | |
|---|-----|
| GAGGANNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN | 420 |
| NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN | 480 |
| NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN | 540 |
| NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN | 600 |
| NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN | 660 |
| NNNNNNNNNN NNNNNNNNNN TCATGTGCAA AAGGGTACAC TCACTGAATG CAATTGATA | 720 |
| TGAAAACCAT ACACAAGCTG ATATAGAAAC ACACCCTCAA CCGAAAAGCA AGCCTAATAA | 780 |
| TTCGGGTTGG GTCGGGTCTA CCATCAATTG TTTTTTCTT TTAACAACCTT TTAATCTCTA | 840 |
| TTTGAGCATG TTGATTCTTG TCTTTTGTGT GTAAGATTTT GGGTTTCGTT TCAGTTGTAA | 900 |
| TAATGAACCA TTGATGTTT GCAATTTCAA GTTCCTATCG ACATGTAGTG ATCTAAAAAA | 960 |

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 305 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

| | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Leu | Arg | Ser | Leu | Leu | Arg | Gly | Leu | Thr | His | Ile | Pro | Arg | Val | Asn |
| 1 | | | | 5 | | | | | 10 | | | | | 15 | |
| Ser | Ala | Gln | Gln | Pro | Ser | Cys | Ala | His | Ala | Arg | Leu | Gln | Phe | Lys | Leu |
| | | | 20 | | | | | 25 | | | | | 30 | | |
| Arg | Ser | Met | Gln | Met | Thr | Leu | Met | Gln | Pro | Ser | Ile | Ser | Ala | Asn | Leu |
| | | 35 | | | | | 40 | | | | | 45 | | | |
| Ser | Arg | Ala | Glu | Asp | Arg | Thr | Asp | His | Met | Arg | Gly | Ala | Ser | Thr | Trp |
| | 50 | | | | | 55 | | | | | 60 | | | | |
| Ala | Gly | Gly | Gln | Ser | Gln | Asp | Glu | Leu | Met | Leu | Lys | Asp | Glu | Cys | Ile |
| 65 | | | | 70 | | | | | 75 | | | | | 80 | |
| Leu | Val | Asp | Val | Glu | Asp | Asn | Ile | Thr | Gly | His | Ala | Ser | Lys | Leu | Glu |
| | | | 85 | | | | | | 90 | | | | | 95 | |
| Cys | His | Lys | Phe | Leu | Pro | His | Gln | Pro | Ala | Gly | Leu | Leu | His | Arg | Ala |
| | | | 100 | | | | | 105 | | | | | | 110 | |

Phe Ser Val Phe Leu Phe Asp Asp Gln Gly Arg Leu Leu Leu Gln Gln
 115 120 125
 Arg Ala Arg Ser Lys Ile Thr Phe Pro Ser Val Trp Thr Asn Thr Cys
 130 135 140
 Cys Ser His Pro Leu His Gly Gln Thr Pro Asp Glu Val Asp Gln Leu
 145 150 155 160
 Ser Gln Val Ala Asp Gly Thr Val Pro Gly Ala Lys Ala Ala Ala Ile
 165 170 175
 Arg Lys Leu Glu His Glu Leu Gly Ile Pro Ala His Gln Leu Pro Ala
 180 185 190
 Ser Ala Phe Arg Phe Leu Thr Arg Leu His Tyr Cys Ala Ala Asp Val
 195 200 205
 Gln Pro Ala Ala Thr Gln Ser Ala Leu Trp Gly Glu His Glu Met Asp
 210 215 220
 Tyr Ile Leu Phe Ile Arg Ala Asn Val Thr Leu Ala Pro Asn Pro Asp
 225 230 235 240
 Glu Val Asp Glu Val Arg Tyr Val Thr Gln Glu Glu Leu Arg Gln Met
 245 250 255
 Met Gln Pro Asp Asn Gly Leu Gln Trp Ser Pro Trp Phe Arg Ile Ile
 260 265 270
 Ala Ala Arg Phe Leu Glu Arg Trp Trp Ala Asp Leu Asp Ala Ala Leu
 275 280 285
 Asn Thr Asp Lys His Glu Asp Trp Gly Thr Val His His Ile Asn Glu
 290 295 300
 Ala
 305

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 293 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Leu Arg Ser Leu Leu Arg Gly Leu Thr His Ile Pro Arg Val Asn
 1 5 10 15

Ser Ala Gln Gln Pro Ser Cys Ala His Ala Arg Leu Gln Phe Lys Leu
 20 25 30
 Arg Ser Met Gln Leu Leu Ser Glu Asp Arg Thr Asp His Met Arg Gly
 35 40 45
 Ala Ser Thr Trp Ala Gly Gly Gln Ser Gln Asp Glu Leu Met Leu Lys
 50 55 60
 Asp Glu Cys Ile Leu Val Asp Val Glu Asp Asn Ile Thr Gly His Ala
 65 70 75 80
 Ser Lys Leu Glu Cys His Lys Phe Leu Pro His Gln Pro Ala Gly Leu
 85 90 95
 Leu His Arg Ala Phe Ser Val Phe Leu Phe Asp Asp Gln Gly Arg Leu
 100 105 110
 Leu Leu Gln Gln Arg Ala Arg Ser Lys Ile Thr Phe Pro Ser Val Trp
 115 120 125
 Thr Asn Thr Cys Cys Ser His Pro Leu His Gly Gln Thr Pro Asp Glu
 130 135 140
 Val Asp Gln Leu Ser Gln Val Ala Asp Gly Thr Val Pro Gly Ala Lys
 145 150 155 160
 Ala Ala Ala Ile Arg Lys Leu Glu His Glu Leu Gly Ile Pro Ala His
 165 170 175
 Gln Leu Pro Ala Ser Ala Phe Arg Phe Leu Thr Arg Leu His Tyr Cys
 180 185 190
 Ala Ala Asp Val Gln Pro Ala Ala Thr Gln Ser Ala Leu Trp Gly Glu
 195 200 205
 His Glu Met Asp Tyr Ile Leu Phe Ile Arg Ala Asn Val Thr Leu Ala
 210 215 220
 Pro Asn Pro Asp Glu Val Asp Glu Val Arg Tyr Val Thr Gln Glu Glu
 225 230 235 240
 Leu Arg Gln Met Met Gln Pro Asp Asn Gly Leu Gln Trp Ser Pro Trp
 245 250 255
 Phe Arg Ile Ile Ala Ala Arg Phe Leu Glu Arg Trp Trp Ala Asp Leu
 260 265 270
 Asp Ala Ala Leu Asn Thr Asp Lys His Glu Asp Trp Gly Thr Val His
 275 280 285
 His Ile Asn Glu Ala
 290

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

50

- (A) LENGTH: 284 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```

Met Ser Val Ser Ser Leu Phe Asn Leu Pro Leu Ile Arg Leu Arg Ser
1           5           10           15

Leu Ala Leu Ser Ser Ser Phe Ser Ser Phe Arg Phe Ala His Arg Pro
          20           25           30

Leu Ser Ser Ile Ser Pro Arg Lys Leu Pro Asn Phe Arg Ala Phe Ser
          35           40           45

Gly Thr Ala Met Thr Asp Thr Lys Asp Ala Gly Met Asp Ala Val Gln
50           55           60

Arg Arg Leu Met Phe Glu Asp Glu Cys Ile Leu Val Asp Glu Thr Asp
65           70           75           80

Arg Val Val Gly His Val Ser Lys Tyr Asn Cys His Leu Met Glu Asn
          85           90           95

Ile Glu Ala Lys Asn Leu Leu His Arg Ala Phe Ser Val Phe Leu Phe
          100          105          110

Asn Ser Lys Tyr Glu Leu Leu Leu Gln Gln Arg Ser Asn Thr Lys Val
          115          120          125

Thr Phe Pro Leu Val Trp Thr Asn Thr Cys Cys Ser His Pro Leu Tyr
          130          135          140

Arg Glu Ser Glu Leu Ile Gln Asp Asn Ala Leu Gly Val Arg Asn Ala
145          150          155          160

Ala Gln Arg Lys Leu Leu Asp Glu Leu Gly Ile Val Ala Glu Asp Val
          165          170          175

Pro Val Asp Glu Phe Thr Pro Leu Gly Arg Met Leu Tyr Lys Ala Pro
          180          185          190

Ser Asp Gly Lys Trp Gly Glu His Glu Leu Asp Tyr Leu Leu Phe Ile
          195          200          205

Val Arg Asp Val Lys Val Gln Pro Asn Pro Asp Glu Val Ala Glu Ile
          210          215          220

Lys Tyr Val Ser Arg Glu Glu Leu Lys Glu Leu Val Lys Lys Ala Asp
225          230          235          240

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Ala Gly Glu Glu Gly Leu Lys Leu Ser Pro Trp Phe Arg Leu Val Val
245 250 255

Asp Asn Phe Leu Met Lys Trp Trp Asp His Val Glu Lys Gly Thr Leu
260 265 270

Val Glu Ala Ile Asp Met Lys Thr Ile His Lys Leu
275 280

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 287 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Ser Ser Ser Met Leu Asn Phe Thr Ala Ser Arg Ile Val Ser Leu
1 5 10 15

Pro Leu Leu Ser Ser Pro Pro Ser Arg Val His Leu Pro Leu Cys Phe
20 25 30

Phe Ser Pro Ile Ser Leu Thr Gln Arg Phe Ser Ala Lys Leu Thr Phe
35 40 45

Ser Ser Gln Ala Thr Thr Met Gly Glu Val Val Asp Ala Gly Met Asp
50 55 60

Ala Val Gln Arg Arg Leu Met Phe Glu Asp Glu Cys Ile Leu Val Asp
65 70 75 80

Glu Asn Asp Lys Val Val Gly His Glu Ser Lys Tyr Asn Cys His Leu
85 . 90 95

Met Glu Lys Ile Glu Ser Glu Asn Leu Leu His Arg Ala Phe Ser Val
100 105 110

Phe Leu Phe Asn Ser Lys Tyr Glu Leu Leu Leu Gln Gln Arg Ser Ala
115 120 125

Thr Lys Val Thr Phe Pro Leu Val Trp Thr Asn Thr Cys Cys Ser His
130 135 140

Pro Leu Tyr Arg Glu Ser Glu Leu Ile Asp Glu Asn Cys Leu Gly Val
145 150 155 160

Arg Asn Ala Ala Gln Arg Lys Leu Leu Asp Glu Leu Gly Ile Pro Ala
165 170 175

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Glu Asp Leu Pro Val Asp Gln Phe Ile Pro Leu Ser Arg Ile Leu Tyr
180 185 190

Lys Ala Pro Ser Asp Gly Lys Trp Gly Glu His Glu Leu Asp Tyr Leu
195 200 205

Leu Phe Ile Ile Arg Asp Val Asn Leu Asp Pro Asn Pro Asp Glu Val
210 215 220

Ala Glu Val Lys Tyr Met Asn Arg Asp Asp Leu Lys Glu Leu Leu Arg
225 230 235 240

Lys Ala Asp Ala Glu Glu Glu Gly Val Lys Leu Ser Pro Trp Phe Arg
245 250 255

Leu Val Val Asp Asn Phe Leu Phe Lys Trp Trp Asp His Val Glu Lys
260 265 270

Gly Ser Leu Lys Asp Ala Ala Asp Met Lys Thr Ile His Lys Leu
275 280 285

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 261 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Thr Gly Pro Pro Pro Arg Phe Phe Pro Ile Arg Ser Pro Val Pro Arg
1 5 10 15

Thr Gln Leu Phe Val Arg Ala Phe Ser Ala Val Thr Met Thr Asp Ser
20 25 30

Asn Asp Ala Gly Met Asp Ala Val Gln Arg Arg Leu Met Phe Glu Asp
35 40 45

Glu Cys Ile Leu Val Asp Glu Asn Asn Arg Val Val Gly His Asp Thr
50 55 60

Lys Tyr Asn Cys His Leu Met Glu Lys Ile Glu Ala Glu Asn Leu Leu
65 70 75 80

His Arg Ala Phe Ser Val Phe Leu Phe Asn Ser Lys Tyr Glu Leu Leu
85 90 95

Leu Gln Gln Arg Ser Lys Thr Lys Val Thr Phe Pro Leu Val Trp Thr
100 105 110

Asn Thr Cys Cys Ser His Pro Leu Tyr Arg Glu Ser Glu Leu Ile Glu
 115 120 125
 Glu Asn Val Leu Gly Val Arg Asn Ala Ala Gln Arg Lys Leu Phe Asp
 130 135 140
 Glu Leu Gly Ile Val Ala Glu Asp Val Pro Val Asp Glu Phe Thr Pro
 145 150 155 160
 Leu Gly Arg Met Leu Tyr Lys Ala Pro Ser Asp Gly Lys Trp Gly Glu
 165 170 175
 His Glu Val Asp Tyr Leu Leu Phe Ile Val Arg Asp Val Lys Leu Gln
 180 185 190
 Pro Asn Pro Asp Glu Val Ala Glu Ile Lys Tyr Val Ser Arg Glu Glu
 195 200 205
 Leu Lys Glu Leu Val Lys Lys Ala Asp Ala Gly Asp Glu Ala Val Lys
 210 215 220
 Leu Ser Pro Trp Phe Arg Leu Val Val Asp Asn Phe Leu Met Lys Trp
 225 230 235 240
 Trp Asp His Val Glu Lys Gly Thr Ile Thr Glu Ala Ala Asp Met Lys
 245 250 255
 Thr Ile His Lys Leu
 260

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 288 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Met Thr Ala Asp Asn Asn Ser Met Pro His Gly Ala Val Ser Ser Tyr
 1 5 10 15
 Ala Lys Leu Val Gln Asn Gln Thr Pro Glu Asp Ile Leu Glu Glu Phe
 20 25 30
 Pro Glu Ile Ile Pro Leu Gln Gln Arg Pro Asn Thr Arg Ser Ser Glu
 35 40 45
 Thr Ser Asn Asp Glu Ser Gly Glu Thr Cys Phe Ser Gly His Asp Glu
 50 55 60

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| | | | | | | | | | | | | | | | | | | | |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Glu | Gln | Ile | Lys | Leu | Met | Asn | Glu | Asn | Cys | Ile | Val | Leu | Asp | Trp | Asp | 65 | 70 | 75 | 80 |
| Asp | Asn | Ala | Ile | Gly | Ala | Gly | Thr | Lys | Lys | Val | Cys | His | Leu | Met | Glu | 85 | 90 | 95 | |
| Asn | Ile | Glu | Lys | Gly | Leu | Leu | His | Arg | Ala | Phe | Ser | Val | Phe | Ile | Phe | 100 | 105 | 110 | |
| Asn | Glu | Gln | Gly | Glu | Leu | Leu | Leu | Gln | Gln | Arg | Ala | Thr | Glu | Lys | Ile | 115 | 120 | 125 | |
| Thr | Phe | Pro | Asp | Leu | Trp | Thr | Asn | Thr | Cys | Cys | Ser | His | Pro | Leu | Cys | 130 | 135 | 140 | |
| Ile | Asp | Asp | Glu | Leu | Gly | Leu | Lys | Gly | Lys | Leu | Asp | Asp | Lys | Ile | Lys | 145 | 150 | 155 | 160 |
| Gly | Ala | Ile | Thr | Ala | Ala | Val | Arg | Lys | Leu | Asp | His | Glu | Leu | Gly | Ile | 165 | 170 | 175 | |
| Pro | Glu | Asp | Glu | Thr | Lys | Thr | Arg | Gly | Lys | Phe | His | Phe | Leu | Asn | Arg | 180 | 185 | 190 | |
| Ile | His | Tyr | Met | Ala | Pro | Ser | Asn | Glu | Pro | Trp | Gly | Glu | His | Glu | Ile | 195 | 200 | 205 | |
| Asp | Tyr | Ile | Leu | Phe | Tyr | Lys | Ile | Asn | Ala | Lys | Glu | Asn | Leu | Thr | Val | 210 | 215 | 220 | |
| Asn | Pro | Asn | Val | Asn | Glu | Val | Arg | Asp | Phe | Lys | Trp | Val | Ser | Pro | Asn | 225 | 230 | 235 | 240 |
| Asp | Leu | Lys | Thr | Met | Phe | Ala | Asp | Pro | Ser | Tyr | Lys | Phe | Thr | Pro | Trp | 245 | 250 | 255 | |
| Phe | Lys | Ile | Ile | Cys | Glu | Asn | Tyr | Leu | Phe | Asn | Trp | Trp | Glu | Gln | Leu | 260 | 265 | 270 | |
| Asp | Asp | Leu | Ser | Glu | Val | Glu | Asn | Asp | Arg | Gln | Ile | His | Arg | Met | Leu | 275 | 280 | 285 | |

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 456 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Met Asp Thr Leu Leu Lys Thr Pro Asn Leu Glu Phe Leu Pro His Gly
 1 5 10 15
 Phe Val Lys Ser Phe Ser Lys Phe Gly Lys Cys Glu Gly Val Cys Val
 20 25 30
 Lys Ser Ser Ala Leu Leu Glu Leu Val Pro Glu Thr Lys Lys Glu Asn
 35 40 45
 Leu Asp Phe Glu Leu Pro Met Tyr Asp Pro Ser Lys Gly Val Val Asp
 50 55 60
 Leu Ala Val Val Gly Gly Gly Pro Ala Gly Leu Ala Val Ala Gln Gln
 65 70 75 80
 Val Ser Glu Ala Gly Leu Ser Val Cys Ser Ile Asp Pro Pro Lys Leu
 85 90 95
 Ile Trp Pro Asn Asn Tyr Gly Val Trp Val Asp Glu Phe Glu Ala Met
 100 105 110
 Asp Leu Leu Asp Cys Leu Asp Ala Thr Trp Ser Gly Ala Val Tyr Ile
 115 120 125
 Asp Asp Thr Lys Asp Leu Arg Pro Tyr Gly Arg Val Asn Arg Lys Gln
 130 135 140
 Leu Lys Ser Lys Met Met Gln Lys Cys Ile Asn Gly Val Lys Phe His
 145 150 155 160
 Gln Ala Lys Val Ile Lys Val Ile His Glu Glu Lys Ser Met Leu Ile
 165 170 175
 Cys Asn Asp Gly Thr Ile Gln Ala Thr Val Val Leu Asp Ala Thr Gly
 180 185 190
 Phe Ser Arg Leu Val Gln Tyr Asp Lys Pro Tyr Asn Pro Gly Tyr Gln
 195 200 205
 Val Ala Tyr Gly Ile Leu Ala Glu Val Glu Glu His Pro Phe Asp Lys
 210 215 220
 Met Val Phe Met Asp Trp Arg Asp Ser His Leu Asn Asn Glu Leu Lys
 225 230 235 240
 Glu Arg Asn Ser Ile Pro Thr Phe Leu Tyr Ala Met Pro Phe Ser Ser
 245 250 255
 Asn Arg Ile Phe Leu Glu Glu Thr Ser Leu Val Ala Arg Pro Gly Leu
 260 265 270
 Arg Met Asp Asp Ile Gln Glu Arg Met Val Ala Arg Leu His Leu Gly
 275 280 285
 Ile Lys Val Lys Ser Ile Glu Glu Asp Glu His Cys Val Ile Pro Met
 290 295 300

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Gly Gly Pro Leu Pro Val Leu Pro Gln Arg Val Val Gly Ile Gly Gly
 305 310 315 320
 Thr Ala Gly Met Val His Pro Ser Thr Gly Tyr Met Val Ala Arg Thr
 325 330 335
 Leu Ala Ala Ala Pro Val Val Ala Asn Ala Ile Ile Tyr Leu Gly Ser
 340 345 350
 Glu Ser Ser Gly Glu Leu Ser Ala Glu Val Trp Lys Asp Leu Trp Pro
 355 360 365
 Ile Glu Arg Arg Arg Gln Arg Glu Phe Phe Cys Phe Gly Met Asp Ile
 370 375 380
 Leu Leu Lys Leu Asp Leu Pro Ala Thr Arg Arg Phe Phe Asp Ala Phe
 385 390 395 400
 Phe Asp Leu Glu Pro Arg Tyr Trp His Gly Phe Leu Ser Ser Arg Leu
 405 410 415
 Phe Leu Pro Glu Leu Ile Val Phe Gly Leu Ser Leu Phe Ser His Ala
 420 425 430
 Ser Asn Thr Ser Arg Glu Ile Met Thr Lys Gly Thr Pro Leu Val Met
 435 440 445
 Ile Asn Asn Leu Leu Gln Asp Glu
 450 455

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 524 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Glu Cys Val Gly Ala Arg Asn Phe Ala Ala Met Ala Val Ser Thr
 1 5 10 15
 Phe Pro Ser Trp Ser Cys Arg Arg Lys Phe Pro Val Val Lys Arg Tyr
 20 25 30
 Ser Tyr Arg Asn Ile Arg Phe Gly Leu Cys Ser Val Arg Ala Ser Gly
 35 40 45
 Gly Gly Ser Ser Gly Ser Glu Ser Cys Val Ala Val Arg Glu Asp Phe
 50 55 60

Ala Asp Glu Glu Asp Phe Val Lys Ala Gly Gly Ser Glu Ile Leu Phe
 65 70 75 80
 Val Gln Met Gln Gln Asn Lys Asp Met Asp Glu Gln Ser Lys Leu Val
 85 90 95
 Asp Lys Leu Pro Pro Ile Ser Ile Gly Asp Gly Ala Leu Asp His Val
 100 105 110
 Val Ile Gly Cys Gly Pro Ala Gly Leu Ala Leu Ala Ala Glu Ser Ala
 115 120 125
 Lys Leu Gly Leu Lys Val Gly Leu Ile Gly Pro Asp Leu Pro Phe Thr
 130 135 140
 Asn Asn Tyr Gly Val Trp Glu Asp Glu Phe Asn Asp Leu Gly Leu Gln
 145 150 155 160
 Lys Cys Ile Glu His Val Trp Arg Glu Thr Ile Val Tyr Leu Asp Asp
 165 170 175
 Asp Lys Pro Ile Thr Ile Gly Arg Ala Tyr Gly Arg Val Ser Arg Arg
 180 185 190
 Leu Leu His Glu Glu Leu Leu Arg Arg Cys Val Glu Ser Gly Val Ser
 195 200 205
 Tyr Leu Ser Ser Lys Val Asp Ser Ile Thr Glu Ala Ser Asp Gly Leu
 210 215 220
 Arg Leu Val Ala Cys Asp Asp Asn Asn Val Ile Pro Cys Arg Leu Ala
 225 230 235 240
 Thr Val Ala Ser Gly Ala Ala Ser Gly Lys Leu Leu Gln Tyr Glu Val
 245 250 255
 Gly Gly Pro Arg Val Cys Val Gln Thr Ala Tyr Gly Val Glu Val Glu
 260 265 270
 Val Glu Asn Ser Pro Tyr Asp Pro Asp Gln Met Val Phe Met Asp Tyr
 275 280 285
 Arg Asp Tyr Thr Asn Glu Lys Val Arg Ser Leu Glu Ala Glu Tyr Pro
 290 295 300
 Thr Phe Leu Tyr Ala Met Pro Met Thr Lys Ser Arg Leu Phe Phe Glu
 305 310 315 320
 Glu Thr Cys Leu Ala Ser Lys Asp Val Met Pro Phe Asp Leu Leu Lys
 325 330 335
 Thr Lys Leu Met Leu Arg Leu Asp Thr Leu Gly Ile Arg Ile Leu Lys
 340 345 350
 Thr Tyr Glu Glu Glu Trp Ser Tyr Ile Pro Val Gly Gly Ser Leu Pro
 355 360 365

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Asn Thr Glu Gln Lys Asn Leu Ala Phe Gly Ala Ala Ala Ser Met Val
 370 375 380

His Pro Ala Thr Gly Tyr Ser Val Val Arg Ser Leu Ser Glu Ala Pro
 385 390 395 400

Lys Tyr Ala Ser Val Ile Ala Glu Ile Leu Arg Glu Glu Thr Thr Lys
 405 410 415

Gln Ile Asn Ser Asn Ile Ser Arg Gln Ala Trp Asp Thr Leu Trp Pro
 420 425 430

Pro Glu Arg Lys Arg Gln Arg Ala Phe Phe Leu Phe Gly Leu Ala Leu
 435 440 445

Ile Val Gln Phe Asp Thr Glu Gly Ile Arg Ser Phe Phe Arg Thr Phe
 450 455 460

Phe Arg Leu Pro Lys Trp Met Trp Gln Gly Phe Leu Gly Ser Thr Leu
 465 470 475 480

Thr Ser Gly Asp Leu Val Leu Phe Ala Leu Tyr Met Phe Val Ile Ser
 485 490 495

Pro Asn Asn Leu Arg Lys Gly Leu Ile Asn His Leu Ile Ser Asp Pro
 500 505 510

Thr Gly Ala Thr Met Ile Lys Thr Tyr Leu Lys Val
 515 520

Claims

1. An isolated eukaryotic enzyme having the amino acid sequence of SEQ ID NO: 2, 4, 14, 15, 16 or 18.
2. An isolated eukaryotic enzyme of Claim 1 which is a cyclase enzyme having the amino acid sequence of SEQ ID NO: 2.
3. An isolated DNA sequence comprising a gene encoding the eukaryotic cyclase of Claim 2.
4. The isolated DNA sequence according to Claim 3, having the nucleic acid sequence of SEQ ID NO: 1.
5. An expression vector comprising the DNA sequence of Claim 3.
6. The expression vector according to Claim 5 which is pATeps deposited with the American Type Culture Collection on March 4, 1996 under accession number 98005.
7. A host containing the expression vector of Claim 5.
8. A host containing the expression vector of Claim 6.
9. An isolated eukaryotic enzyme of Claim 1, which is an isopentenyl isomerase (IPP) enzyme having the amino acid sequence of SEQ ID NOS: 14, 15, 16 or 18.
10. An isolated DNA sequence comprising a gene encoding the IPP enzyme of Claim 9.
11. The isolated DNA sequence of Claim 10, having the nucleic acid sequence of SEQ ID NOS: 9, 10, 11 or 12.
12. An expression vector comprising the DNA sequence of Claim 10.

13. The expression vector of Claim 11 which is PHP05, pMDP1, pATDP7 or PHP04, deposited with the American Type Culture Collection on March 4, 1996 under accession Nos. 98000, 98001, 98002 or 98004.

14. A host containing the expression vector of Claim 12.

15. The isolated eukaryotic enzyme of Claim 1, which is β -carotene hydroxylase enzyme having the amino acid sequence of SEQ ID NO: 4.

16. An isolated DNA sequence comprising a gene encoding the β -carotene hydroxylase enzyme of Claim 15.

17. The isolated DNA sequence according to Claim 16, having the nucleic acid sequence of SEQ ID NO: 3.

18. An expression vector comprising the DNA sequence of Claim 16.

19. The expression vector according to Claim 18 which is pAT0HB deposited with the American Type Culture Collection on March 4, 1996 under accession number 98003.

20. A host containing the expression vector of Claim 18.

21. A host containing the expression vector of Claim 19.

22. A DNA sequence which, when incorporated into a prokaryotic host, results in the expression of an eukaryotic carotenoid biosynthetic enzyme,

wherein said DNA sequence comprises a truncated portion of the naturally occurring DNA sequence encoding said eukaryotic carotenoid biosynthetic enzyme, wherein said

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truncated portion comprises said natural sequence minus at least one codon at the 5' terminus.

23. The DNA sequence of Claim 22, wherein said eukaryotic carotenoid biosynthetic enzyme is β -carotene hydroxylase.

24. The DNA sequence of Claim 23, which is a BalIII - 3' end exofragment of SEQ ID NO: 3 fused to a 5' ATG start codon.

25. A method for screening for eukaryotic genes involved in carotenoid biosynthesis, metabolism or degradation comprising the steps of:

engineering of a prokaryotic host which accumulates a carotenoid or carotenoid precursor or which is deficient in an enzyme of the carotenoid pathway;

transforming said host with DNA which may contain an eukaryotic carotenoid biosynthetic gene;

culturing said transformed host to obtain colonies; and

screening for colonies exhibiting a different visual appearance than colonies of the untransformed host.

26. The method of Claim 25, wherein said prokaryotic host is *E. coli*.

27. A method for producing a carotenoid, comprising the steps of:

transforming a host with DNA which comprises a eukaryotic carotenoid biosynthetic gene;

culturing said host for a time sufficient for said host to produce said carotenoid; and

collecting said carotenoid from the host.

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28. The method of Claim 26, wherein said DNA further comprises a isopentyl pyrophosphate isomerase gene.

29. A method for inhibiting carotenoid biosynthesis in a host, comprising the steps of:

transforming said host with antisense DNA to a eukaryotic carotenoid biosynthesis gene; and

culturing said host.

30. A method for increasing production of a secondary metabolite of isopentyl pyrophosphate (IPP) by a host, comprising the steps of:

transforming said host with DNA that comprises an isopentyl pyrophosphate isomerase gene; and

culturing said host for a time sufficient to produce said secondary metabolite; and

recovering said secondary metabolite from said host.

31. The method of Claim 30, wherein said secondary metabolite is a carotenoid.

32. A method for screening for secondary metabolites, comprising:

engineering a host which accumulates a secondary metabolite or secondary metabolite precursor of isopentyl pyrophosphate (IPP); and

transforming said host with DNA that may contain an IPP isomerase gene; and

culturing said host for a time sufficient to accumulate said secondary metabolite or precursor; and

screening for said secondary metabolite or precursor.

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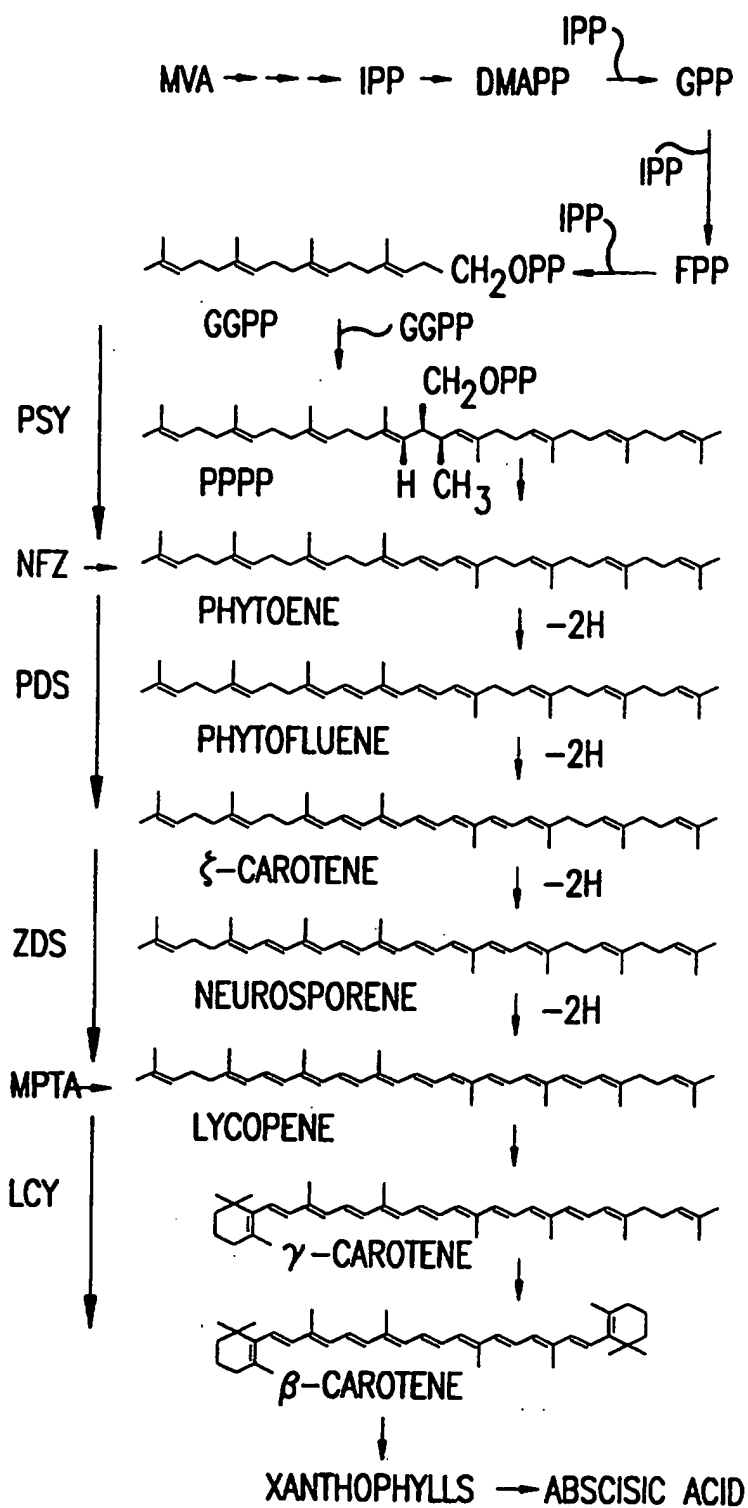


FIG.1

SUBSTITUTE SHEET (RULE 26)

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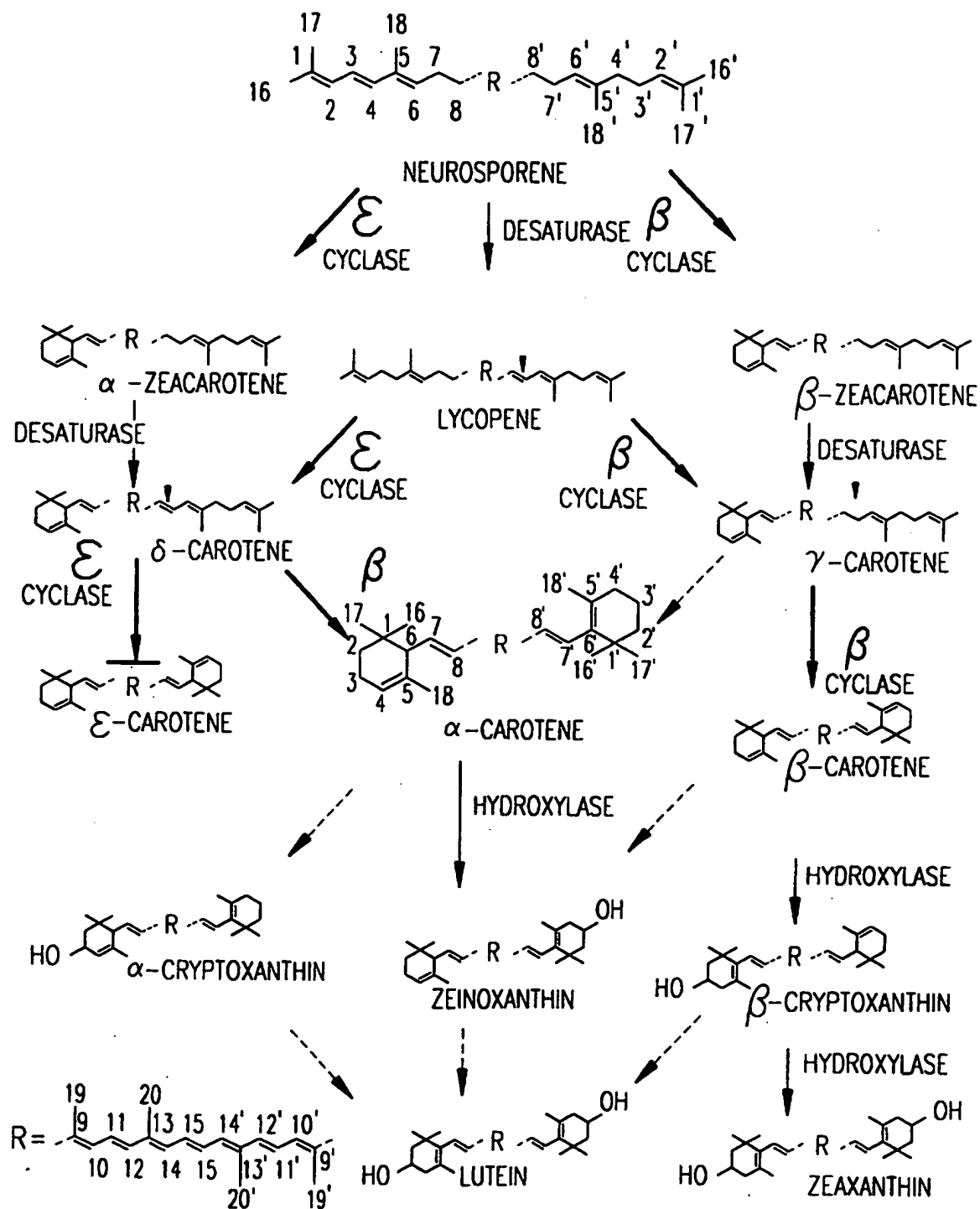


FIG.2

SUBSTITUTE SHEET (RULE 26)

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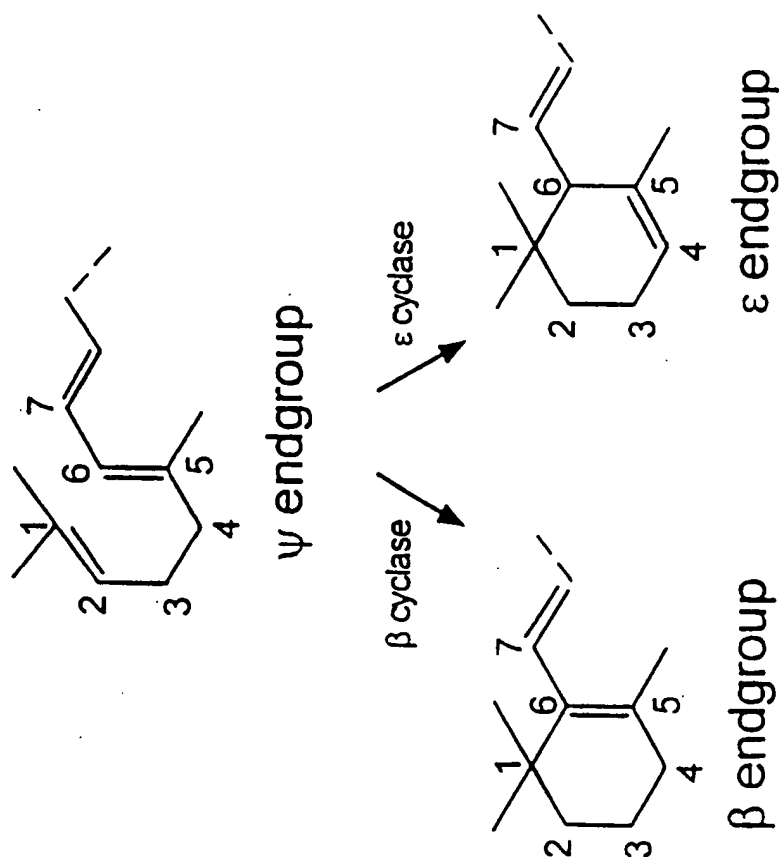


FIG.3

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acaaaaggaaataattag attcctctttctgcttgctataccttgata 48

gaacaatataacaatggtgtaagtcttctc gctgtattcgaaattatttgaggaggaaa 108

atggagtgtgttggggctaggaatttcgca gcaatggcggtttcaacatttccgcatgg 168
1 M E C V G A R N F A A M A V S T F P S W

agttgtcgaaggaaatttccagtggttaag agatacagctataggaatattcgtttcggt 228
21 S C R R K F P V V K R Y S Y R N I R F G

ttgtgtagtgtcagagctagcggcgccgga agttccggtagtgtgagagttgtgtagcgggtg 288
41 L C S V R A S G G G S S G S E S C V A V

agagaagatttgcgtgacgaagaagatttt gtgaaagctggtggttctgagattctattt 348
61 R E D F A D E E D F V K A G G S E I L F

gttcaaatgcagcagaacaaagatatggat gaacagtctaagcttggtgataagttgcct 408
81 V Q M Q Q N K D M D E Q S K L V D K L P

cctatatcaattggtgatggtgctttggat catgtggttattggttggtcctgctggt 468
101 P I S I G D G A L D H V V I G C G P A G

ttagccttggtgcagaatcagctaagctt ggattaaaagttggactcattggtccagat 528
121 L A L A A E S A K L G L K V G L I G P D

cttccttttactaacaattacggtggttgg gaagatgaattcaatgatcttgggctgcaa 588
141 L P F T N N Y G V W E D E F N D L G L Q

aatgtattgagcatgtttggagagagact attgtgtatctggatgatgacaagcctatt 648
161 K C I E H V W R E T I V Y L D D D K P I

accattggccgtgcttatggaagagtagt cgacgtttgctccatgaggagcttttgagg 708
181 T I G R A Y G R V S R R L L H E E L L R

aggtgtgtcaggtcaggtgtctcgtaacctt agctcgaaagttgacagcataacagaagct 768
201 R C V E S G V S Y L S S K V D S I T E A

tctgatggccttagacttggtgcttgtagac gacaataacgtcattccctgcaggcttgcc 828
221 S D G L R L V A C D D N N V I P C R L A

actgttgcttctggagcagcttcgggaaag ctcttgcaatacgaagttggtggacctaga 888
241 T V A S G A A S G K L L Q Y E V G G P R

FIG.4A

SUBSTITUTE SHEET (RULE 26)

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gtctgtgtgcaaactgcatacggcgtggag gttgaggtggaaaatagtccatatgatcca 948
261 V C V Q T A Y G V E V E V E N S P Y D P

gatcaaatggttttcatggattacagagat tatactaacgagaaaagttcggagcttagaa 1008
281 D Q M V F M D Y R D Y T N E K V R S L E

gctgagtatccaacgtttctgtacgccatg cctatgacaaagtcaagactcttcttcgag 1068
301 A E Y P T F L Y A M P M T K S R L F F E

gagacatgtttggcctcaaaagatgtcatg ccctttgatttgctaaaaacgaagctcatg 1128
321 E T C L A S K D V M P F D L L K T K L M

ttaagattagatacactcgggaattcgaatt ctaaagacttacgaagaggagtggtcctat 1188
341 L R L D T L G I R I L K T Y E E E W S Y

atcccagttgggtggttccttgccaaacacc gaacaaaagaatctcgcctttggtgctgcc 1248
361 I P V G G S L P N T E Q K N L A F G A A

gctagcatggtacatcccgcaacaggctat tcagttgtgagatctttgtctgaagctcca 1308
381 A S M V H P A T G Y S V V R S L S E A P

aatatgcatcagtcacgcagagatacta agagaagagactaccaaacagatcaacagt 1368
401 K Y A S V I A E I L R E E T T K Q I N S

aatatttcaagacaagcttgggatacttta tggccaccagaaaaggaaaagacagagagca 1428
421 N I S R Q A W D T L W P P E R K R Q R A

aatatttcaagacaagcttgggatacttta tggccaccagaaaaggaaaagacagagagca 1488
441 F F L F G L A L I V Q F D T E G I R S F

ttccgtacttttctccgccttccaaaatgg atgtggcaagggtttctaggatcaacatta 1548
461 F R T F F R L P K W M W Q G F L G S T L

acatcaggagatctcgttctctttgcttta tacatggttcgtcatttcaccaaacaatttg 1608
481 T S G D L V L F A L Y M F V I S P N N L

agaaaagggtctcatcaatcatctcatctct gatccaaccggagcaaccatgataaaaacc 1668
501 R K G L I N H L I S D P T G A T M I K T

tatctcaaagtatgattttacttatcaactc ttaggtttgtgtatatatatgttgatttat 1728
521 Y L K V

FIG.4B

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ctgaataatcgatcaaagaatggtatgtgg gttactaggaagttgaaacaaacatgtat 1778
agaatctaaggagtgatcgaaatggagatg gaaacgaaaagaaaaaatcagtcctttggtt 1848
ttgtgggttagtg 1860

FIG.4C

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1 gctctttctc ctctcctct accgatttcc gactccgcct cccgaaatcc
51 ttatccggat tctctccgtc tcttcgattt aaacgctttt ctgtctgtta
101 cgtcgtcgaa gaacggagac agaattctcc gattgagaac gatgagagac
151 cggagagcac gagctccaca aacgctatag acgctgagta tctggcggtg
201 cgtttggcgg agaaattgga gaggaagaaa tcggagaggt ccacttatct
251 aatcgtgct atgttgtcga gctttggtat cacttctatg gctgttatgg
301 ctgtttacta cagattctct tggcaaatgg agggaggtga gatctcaatg
351 ttggaaatgt ttggtacatt tgctctctct gttggtgctg ctgttggtat
401 ggaattctgg gcaagatggg ctcatagagc tctgtggcac gcttctctat
451 ggaatatgca tgagtcacat cacaaccaa gagaaggacc gtttgagcta
501 aacgatgttt ttgctatagt gaacgctggt ccagcgattg gtctcctctc
551 ttatggattc ttcaataaag gactcgttcc tggctctctgc tttggcgccg
601 ggtaggcat aacggtgttt ggaatcgctt acatgtttgt ccacgatggt
651 ctctgcaca agcgtttccc tgtaggtccc atcgccgacg tcccttacct
701 ccgaaaggtc gccgccgctc accagctaca tcacacagac aagttcaatg
751 gtgtaccata tggactgttt cttggacca aggaattgga agaagttgga
801 ggaaatgaag agttagataa ggagattagt cggagaatca aatcatacaa
851 aaaggcctcg ggctccgggt cgagttcgag ttcttgactt taaacaagtt
901 ttaaatccca aattcttttt ttgtcttctg tcattatgat catcttaaga
951 cggctc

FIG.5

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A.thal. 64 SFSS SSTDFRLRLP KSLSGFSPSL RFKRFSVCYW VEERRQNSPI ENDERPESTS STNAIDAEYL

A.thal. 144 ALRLAEKLER KKSERSTYLI AAMLSSFGIT SMAMVAVYYR FSWQMEGGEI SMLEMFGTFA LSVGAAGVME FWARWAHRAL
 Alical. MTQFL IIVATVLVME LTAYSVHRWI
 A.aurant. MTNFI IIVATVLVME LTAYSVHRWI
 E.herb. ML.NSL IIVILSVIAME GIAAFTHRYI
 E.ured. MLWIWAL IVFVTVIGME VIAALAHKYI

CONSENSUS ----- f - - v - - - - - ME - - A - - - - - Hr - -

PREDICTED TM HELIX PREDICTED TM HELIX

A.thal. 224 WHASL.WNMH ESHHKPREGP FELNDVFAIV NAGPAIGLLS YGFFNKGLVP GLCFGAGLGI TVFGIAYMFV HDGLVHKRFP
 Alical. MHGPLGWGWH KSHHEEHDHA LEKNDLYGVV FAVLATILFT VGAYMWPVLW WI.....ALGM TVYGLIYFIL HDGLVHQRWP
 A.aurant. MHGPLGWGWH KSHHEEHDHA LEKNDLYGLV FAVIATVLFT VGTWAPVLW WI.....ALGM TVYGLIYFVL HDGLVHQRWP
 E.herb. MHG.WGWRWH ESHHTPRKGV FELNDLFAVV FAGVAIALIA VGTAGVWPLQ WI.....GCGM TVYGLLYFLV HDGLVHQRWP
 E.ured. MHG.WGWRWH LSHHEPRKGA FEVNDLYAVV FAALSILLIY LGSTGMWPLQ WI.....GAGM TAYGLLYFMV HDGLVHQRWP

CONSENSUS -H--I-W--H -SHH-pr-g- fE-ND--a-V -A--ai-L-- -G----- -gIg- Tv-G--Y--v HDGLVH-R-P

PREDICTED TM HELIX PREDICTED TM HELIX

FIG.6A

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| | | | | | | | | | | |
|-----------|-------------|-------------|------------|-------------|------------|------------|-------------|------------|-------------|-----|
| A.thal. | VGPIADVPYL | RKVAAAHQLH | HT. | DKFNGV | PYGLFLGPKE | LEEVGGNEEL | DKEISRRIKS | YKKASGSGSS | SSS* | 301 |
| Alcal. | FRYIPRRGYF | RRLYQAHRLH | HAVEGRDHCV | SFGFIYAPP. | VDKLKQDLKR | SGVLRPQDER | PS* | | | |
| A.aurant. | FRYIPRKGYA | RRLYQAHRLH | HAVEGRDHCV | SFGFIYAPP. | VDKLKQDLKM | SGVLRAEAE | RT* | | | |
| E.herb. | FHWIPRRGYL | KRLYVAHRLH | HAVRGREGCV | SFGFIYARK. | PADLQAILRE | RHGRPPKRDA | AKDRPDAAASP | SSSSPE* | | |
| E.ured. | FRYIPRKGYL | KRLYMAHRMH | HAVRGKEGCV | SFGFLYAPP. | LSKLQATLRE | RHG. | ARAGA | ARDAQGGEDE | PASGK* | |
| CONSENSUS | ---I-----YI | r-----AH-TH | H-----V | --G-----p-- | ----- | ----- | ----- | ----- | -----S----- | |

FIG.6B

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1 ccacgggtcc gcctccccgt ttttttccga tccgatctcc ggtgccgag
51 actcagctgt ttgttcgcgc tttctcagcc gtcaccatga ccgattctaa
101 cgatgctgga atggatgctg ttcagagacg actcatgttt gaagacgaat
151 gcattctcgt tgatgaaaat aatcgtgtgg tgggacatga cactaagtat
201 aactgtcatc tgatggaaaa gattgaagct gagaattttac ttcacagagc
251 tttcagtggtg tttttattca actccaagta tgagttgctt ctccagcaac
301 ggtcaaaaac aaaggttact tcccacttg tgtggacaaa cacttgttgc
351 agccatcctc ttaccgtga atccgagctt attgaagaga atgtgcttgg
401 tgtaagaaat gccgcacaaa ggaagctttt cgatgagctc ggtattgtag
451 cagaagatgt accagtcgat gagttcactc ccttgggacg catgctttac
501 aaggcacctt ctgatgggaa atggggagag cacgaagtgg actatctact
551 cttcatcgtg cgggatgtga agcttcaacc aaaccagat gaagtggctg
601 agatcaagta cgtgagcagg gaagagctta aggagctggt gaagaaagca

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FIG. 7A

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651 gatgctggcg atgaagctgt gaaactatct ccatggttca gattggtggt
701 ggataaatttc ttgatgaagt ggtgggatca tgttgagaaa ggaactatca
751 ctgaagctgc agacatgaaa accattcaca agctctgaac tttccataag
801 ttttggatct tcccctccc ataataaat taagagatga gacttttatt
851 gattacagac aaaactggca acaaaatcta ttcctaggat tttttttgc
901 tttttattta cttttgattc atctctagtt tagttttcat cttaaaaaa
951 aaaa

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FIG.7B

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1 caccaatgct tgtttcttct ttatttaatc tccattgat tcgcctcaga
51 tctctcgctc tttcgtcttc ttttctctct ttcgatttg cccatcgctc
101 TCTGTCATCG ATTCACCGA GAAAGTTACC GAATTTTCGT GCTTCTCTG
151 GTACCGCTAT GACAGATACT AAAGATGCTG GTATGGATGC TGTTCAGAGA
201 CGTCTCATGT TTGAGGATGA ATGCATTCTT GTTGATGAAA CTGATCGTGT
251 TGTGGGGCAT GTCAGCAAGT ATAATTGTCA TCTGATGGAA AATATTGAAG
301 CCAAGAATT GCTGCACAGG GCTTTTAGTG TATTTTATT CAACTCGAAG
351 TATGAGTTGC TTCTCCAGCA AAGTCAAAC ACAAAGGTA CGTCCCTCT
401 AGTGTGGACT AACACTTGTT GCAGCCATCC TCTTTACCGT GAATCAGAGC
451 TTATCCAGGA CAATGCACTA GGTGTGAGGA ATGCTGCACA AAGAAAGCTT
501 CTCGATGAGC TTGGTATTGT AGCTGAAGAT GTACCAGTCG ATGAGTTCAC

FIG.8A

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551 TCCCTTGGGA CGTATGCTGT ACAAGGCTCC TTCTGATGGC AAATGGGGAG
601 AGCATGAACT TGATTACTTG CTCTTCATCG TGCAGAGACGT GAAGGTTCAA
651 CCAAACCCAG ATGAAGTAGC TGAGATCAAG TATGTGAGCC GGAAGAGCT
701 GAAGGAGCTG GTGAAGAAAG CAGATGCAGG TGAGGAAGGT TTGAAACTGT
751 CACCATGGTT CAGATTGGTG GTGGACAATT TCTTGATGAA GTGGTGGGAT
801 CATGTTGAGA AAGGAACTTT GGTGAAGCT ATAGACATGA AAACCATCCA
851 CAAACTCTGA ACATCTTTTT TTAAAGTTTT TAAATCAATC AACTTTCTCT
901 TCATCATTTT TATCTTTTCG ATGATAATAA TTTGGGATAT GTGAGACACT
951 TACAAAACCT CCAAGCACCT CAGGCAATAA TAAAGTTTGC GGCCCG

FIG.8B

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1 CTCGGTAGCT GGCCACAATC GCTATTGGA ACCTGGCCCG GCGCAGTCC
51 GATGCCGCGA TGCTTCGTTC GTTGCTCAGA GGCCTCAGC ATATCCCCCG
101 CGTGAACCTC GCCCAGCAGC CCAGCTGTGC ACACGCGCGA CTCCAGTTTA
151 AGCTCAGGAG CATGCAGATG ACGCTCATGC AGCCCAGCAT CTCAGCCAAT
201 CTGTCGCGCG CCGAGGACCG CACAGACCAC ATGAGGGGTG CAAGCACCTG
251 GGCAGGCGGG CAGTCGCAGG ATGAGCTGAT GCTGAAGGAC GAGTGCATCT
301 TGGTGGATGT TGAGGACAAC ATCAGAGGCC ATGCCAGCAA GCTGGAGTGT
351 CACAAGTTCC TACCACATCA GCCTGCAGGC CTGCTGCACC GGGCCTTCTC
401 TGTGTTCCCTG TTTGACGATC AGGGGCGACT GCTGCTGCAA CAGCGTGCAC
451 GCTCAAAAAT CACCTTCCCA AGTGTGTGGA CGAACACCTG CTGCAGCCAC
501 CCTTTACATG GGCAGACCCC AGATGAGGTG GACCAACTAA GCCAGGTGGC
551 CGACGGAACA GTACCTGGCG CAAAGGCTGC TGCCATCCGC AAGTTGGAGC
601 ACGAGCTGGG GATACCAGCG CACCAGCTGC CGGCAAGCGC GTTTCGCTTC

FIG.9A

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651 CTCACGCGTT TGCACACTG TGCCGCGGAC GTGCAGCCAG CTGCGACACA
701 ATCAGCGCTC TGGGGCGAGC ACGAAATGGA CTACATCTTG TTCATCCGGG
751 CCAACGTCAC CTTGGCGCCC AACCCTGACG AGGTGGACGA AGTCAGGTAC
801 GTGACGCAAG AGGAGCTGCG GCAGATGATG CAGCCGGACA ACGGGCTGCA
851 ATGGTCGCCG TGGTTTCGCA TCATCGCCGC GCGCTTCCTT GAGCGTTGGT
901 GGGCTGACCT GGACGCGGCC CTAACACTG ACAACACGA GGATTGGGA
951 ACGGTGCATC ACATCAACGA AGCGTGAAAG CAGAAGCTGC AGGATGTGAA
1001 GACACGTCAT GGGGTGGAAT TCGGTACTTG GCAGCTTCGT ATCTCCCTTT
1051 TCTGAGACTG AACCTGCAGT CAGGTCCCAC AAGGTCAGGT AAAATGGCTC
1101 GATAAAATGT ACCGTCACTT TTTGTCGCGT ATACTGAACT CCAAGAGGTC
1151 AAAAAAAAAA AAAA

FIG.9B

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1 CTCGGTAGCT GGCCACAATC GCTATTGGA ACCTGGCCCG GCGGCAGTCC
51 GATGCCGCGA TGCTTCGTTT GTTGCTCAGA GGCCTCACGC ATATCCCGCG
101 CGTGAACTCC GCCCAGCAGC CCAGCTGTGC ACACGGCGGA CTCCAGTTTA
151 AGCTCAGGAG CATGCAGCTG CTTTCCGAGG ACCGCACAGA CCACATGAGG
201 GGTGCAAGCA CCTGGGCAGG CGGGCAGTCG CAGGATGAGC TGATGCTGAA
251 GGACGAGTGC ATCTTGGTAG ATGTTGAGGA CAACATCACA GGCCATGCCA
301 GCAAGCTGGA GTGTCACAAG TTCCTACCAC ATCAGCCTGC AGGCCTGCTG
351 CACCGGGCCT TCTCTGTGTT CCTGTTTGAC GATCAGGGGC GACTGCTGCT
401 GCAACAGCGT GCACGCTCAA AAATCACCTT CCCAAGTGTG TGGACGAACA
451 CCTGCTGCAG CCACCCCTTA CATGGGCAGA CCCAGATGA GGTGGACCAA
501 CTAAGCCAGG TGGCCGACGG AACAGTACCT GGCGCAAAGG CTGCTGCCAT

FIG.10A

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551 CCGCAAGTTG GAGCAGGAGC TGGGGATACC AGCGCACCAG CTGCCGGCAA
601 GCGCGTTTCG CTTCTCAGC CGTTTGCACT ACTGTGCCGC GGACGTGCAG
651 CCAGCTGCGA CACAATCAGC GCTCTGGGC GAGCAGGAAA TGGACTACAT
701 CTTGTTTCATC CGGGCCAACG TCACCTTGGC GCCCAACCTT GACGAGGTGG
751 ACGAAGTCAG GTACGTGACG CAAGAGGAGC TCGGCGAGAT GATGCAGCCG
801 GACAACGGGC TTCAATGGTC GCCGTGGTTT CGCATCATCG CCGCGGCTT
851 CCTTGAGCGT TGGTGGGCTG ACCTGGACGC GGCCCTAAAC ACTGACAAAC
901 ACGAGGATTG GGAACGGTG CATCACATCA ACGAAGCGTG AAGGCAGAAG
951 CTGCAGGATG TGAAGACACG TCATGGGGTG GAATTGCGTA CTTGGCAGCT
1001 TCGTATCTCC TTTTCTGAG ACTGAACCTG CAGAGCTAGA GTCAATGGTG
1051 CATCATATTC ATCGTCTCTC TTTTGTTTA GACTAATCTG TAGCTAGAGT
1101 CACTGATGAA TCCTTTACAA CTTTCAAAA AAAA

FIG.10B

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| | | | | | | | |
|----------|-----|------------|------------|------------|-------------|------------|-----|
| HP04 | 1 | MLRSLRLGLT | HIPRVNSAQQ | PSCAHARLQF | KLRSMQMTLM | QPSISANLSR | 50 |
| HP05 | | MLRSLRLGLT | HIPRVNSAQQ | PSCAHARLQF | KLRSMQLL.. | | |
| ADP7 | | MSVSSLEFNL | .LIRLSLA. | LSSSFSSFRF | AHRPLSSIS. | PRKLPNFRAF | |
| C brew. | | MS.SSMLNFT | .ASRIVSLPL | LSSPPSRVHL | PLCFFSPISL | TQRFSAKLTF | |
| ADP5 | | | .TGPPPRFFP | IRSPVPRTQL | FVRAFSAV.. | | |
| S cerev. | | ..MTADNNSM | PHGAVSSYAK | LVQNQTPEDI | LEEFPEIPL | QQRPN...TR | |
| | 51 | AEDRTDHMRG | ASTWAGGQSQ | DEMLLKDECI | LVDVEDNITG | HASKLECHKF | 100 |
| | | SEDRTDHMRG | ASTWAGGQSQ | DEMLLKDECI | LVDVEDNITG | HASKLECHKF | |
| | | S..GTA.MTD | TKDAGMDAVQ | RRLMFEDECI | LVDETDRVVG | HVSKYNCHLM | |
| | | SSQATT.MGE | VVDAGMDAVQ | RRLMFEDECI | LVDENDKVVG | HESKYNCHLM | |
| | |T.MTD | SNDAGMDAVQ | RRLMFEDECI | LVDENNRVVG | HDTKYNCHLM | |
| | | SSETSNDESG | ETCFSGHDEE | QIKLMNENCI | VLDWDDNAIG | AGTKKVCHLM | |
| | 101 | LPHQPAGLLH | RAFSVFLFDD | QGRLLLQORA | RSKITFPSVW | TNTCCSHPLH | 150 |
| | | LPHQPAGLLH | RAFSVFLFDD | QGRLLLQORA | RSKITFPSVW | TNTCCSHPLH | |
| | | ENIEAKNLLH | RAFSVFLFNS | KYELLQORS | NTKVTFPLVW | TNTCCSHPLY | |
| | | EKIESENLLH | RAFSVFLFNS | KYELLQORS | ATKVTFPLVW | TNTCCSHPLY | |
| | | EKIEAENLLH | RAFSVFLFNS | KYELLQORS | TKKVTFPLVW | TNTCCSHPLY | |
| | | ENIE.KGLLH | RAFSVFIFNE | QGELLQORA | TEKITFPDLW | TNTCCSHPLC | |
| | 151 | GQTPDEVDDL | SQVADGTVPG | AKAAAIRKLE | HELGI PAHQL | PA.SAFRFLT | 200 |
| | | GQTPDEVDDL | SQVADGTVPG | AKAAAIRKLE | HELGI PAHQL | PA.SAFRFLT | |
| | | RE..... | SELIQDNALG | VRNAAQRKLL | DELGI VAEDV | PV.DEFTPLG | |

FIG.11A

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RE..... SELIDENCLG VRNAAQRKLL DELGIPAEDL PV.DQFIPLS
 RE..... SELIEENVLG VRNAAQRKLF DELGIVAEDV PV.DEFTPLG
 ID...DELGL KGKLDKIKG AITAAVRKLD HELGIPEDET KTRGKFHFLN

201
 RLHYCAADVQ PAATQSALWG EHEMDYILFIRANVTL APNPDEVDEV 250
 RLHYCAADVQ PAATQSALWG EHEMDYILFIRANVTL APNPDEVDEV
 RMLY..... .KAPSDGKWG EHELDYLLFIVRDVKV QPNPDEVAEI
 RILY..... .KAPSDGKWG EHELDYLLFIIRDVNL DPNPDEVAEV
 RMLY..... .KAPSDGKWG EHEVDYLLFIVRDVKL QPNPDEVAEI
 RIHY..... .MAPSNEPWG EHEIDYILFY KINAKENLTV NPNVNEVRDF

251
 RYVTQEELRQ MMQ.....PDN GLQWSPWFRI IAARFLERWW ADLDAALNTD 300
 RYVTQEELRQ MMQ.....PDN GLQWSPWFRI IAARFLERWW ADLDAALNTD
 KYVSREELKE LVKKADAGEE GLKLSFWFRL VVDNFLMKWW DHVEKGTIVE
 KYMNRDDLKE LVRKADAEAE GVKLSFWFRL VVDNFLFKWW DHVEKGS�KD
 KYVSREELKE LVKKADAGDE AVKLSFWFRL VVDNFLMKWW DHVEKGTITE
 KWVSPNDLKT MF.....ADP SYKFTPWFKI ICENYLFNWW EQLDDLSEVE

301
 KHEDWGTVHH INEA*
 KHEDWGTVHH INEA*
 A.IDMKTIHK L*
 A.ADMKTIHK L*
 A.ADMKTIHK L*
 NDRQ...IHR ML*

FIG.11B

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551 xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx
601 xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx
651 xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx
701 tcactgaatg caatttgata tgaaaaccat acacaagctg atatagaaac
751 acaccctcaa ccgaaaagca agcctaataa ttcgggttgg gtcgggtcta
801 ccatcaattg tttttttctt ttaacaactt ttaatctcta tttgagcatg
851 ttgattcttg tcttttgtgt gtaagatttt gggtttcgtt tcagttgtaa
901 taatgaacca ttgatgggtt gcaatttcaa gttcctatcg acatgtagtg
951 atctaaaaaa

FIG.12B

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1 ccaaaaacaa ctcaaatctc ctccgtcgct ctactccgc catgggtgac
51 gactccggca tggatgctgt tcagcgacgt ctcatgtttg acgatgaatg
101 cattttggtg gatgagtgtg acaatgtggt gggacatgat accaaataca
151 attgtcactt gatggagaag attgaaacag gtaaaatgct gcacagagca
201 ttcagcgttt ttctattcaa ttcaaaatac gagttacttc ttcagcaacg
251 gtctgcaacc aaggtgacat ttcctttagt atggaccaac acctgttgca
301 gccatccact ctacagagaa tccgagcttg tcccgaaac gcctgagaga
351 atgctgcaca gaggaxxxx xxxxxxxxxxxx xxxxxxxxxxxx
401 xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx
451 xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx
501 xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx xxxxxxxxxxxx

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FIG.12A

| | | | | | |
|--|----|----------------|----------------|---|----------------------------------|
| PLANT BETA A. t. EPSILON CONSENSUS | 71 | CYANOBACTERIAL | ENZYMES BEGINS | → | 140 |
| Vk--SsALLE LVPETKKENL DFELP _m YDp. ...S.Kg-VV | | | | | DLAvVGGGPA GLAVAQQVSE AGLSVCSDP |
| VKAGGSEIL. FVQMQQNKDM DEQSKLVDKL PPISIGDGAL | | | | | DHVVIGCGPA GLALAAESAK LGLKVGLIGP |
| VK---S--L- -V-----D- -D-----S----- | | | | | D--V-G-GPA GLA-A-----GL-V--I-P |
| POSSIBLE SUBUNIT INTERACTION DOMAIN | | | | ↑ | DINUCLEOTIDE-BINDING SIGNATURE↑ |

| | | | | | |
|--------------|------------------------------------|------------|-------------|------------|------------|
| PLANT BETA | ..-PKL IVPNN YGVWVDEFEA MDLLDCLDaT | WSGa-VYiDd | -t-KDL-RPY | GRVNRKQLKS | KMmQKCI-NG |
| A.t. EPSILON | DLP...FTNN YGVWEDEFND LGLOKCIHV | WRETIVYLDD | DKPITIGRAY | GRVSRRLHE | ELLRRCVESG |
| CONSENSUS | --p-----NN YGVW-DEF-- | --L--C---- | W-----VY-DD | -----R-Y | -----C--G |

CONSERVED REGION #1

| | | | | | | | | | |
|--------------|------------|------------|------------|------------|------------|------------|------------|------------|-----|
| PLANT BETA | VKFHgaKVik | V:HE.E-kSm | liCnDG-tIQ | AtWLDATGF | SR- | LVQYDK | PYnPGY.QVA | YGIIAEVeeH | 280 |
| A.t. EPSILON | VSYLSSKVDs | ITEADGLRL | VACDDNNVlP | CRLATVASGA | ASGKLlQYEV | GGPRVCVQTA | YGVEVEVENS | | |
| CONSENSUS | V-----KV-- | -----Y-- | --C-D---I- | -----A-G- | ----L-QY-- | -----Q-A | YG---EV--- | | |
| | 211 | | | | | | | | |

FIG. 13A

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281 PLANT BETA
 A.t. EPSILON
 CONSENSUS
 PFD--KMVFH DWRDHL-nn -eLKERNs-i PIFLYAMPFS SNrIFLEETS LVARPGLrmd DIQERMvARL 350
 PYDPQMVFM DYRDY..TNE .KVRSLAEY PIFLYAMPMT KSRLFEEETC LASKDVMPFD LLKTKMLRL
 P-D---MVFM D-RD---N- PIFLYAMP-- --R-F-EET- L-----D -----RL
 ↑ CONSERVED REGION#2 ↑ CONSERVED REGION #3
 351 PLANT BETA
 A.t. EPSILON
 CONSENSUS
 -HLGIkVksI EEDEhCvIPM GGpLPVIPQR VVG:GGTAGm VHPSTGYMVA RTLAAPvVA NAJ:-YLqSe 420
 DTLGIRILKT YEEENSYIPV GGSLPNTQK NLAFGAAASM VHPATGYSVV RSLSEAPKYA SVIAEILREE
 --LGI----- -E-E---IP- GG-LP---Q- ----G--A-M VHP-TGY-V- R-L--AP--A --I---L--E
 ↑ CONSERVED REGION #4 PREDICTED TM HELIX
 421 PLANT BETA
 A.t. EPSILON
 CONSENSUS
 -S-s..G-eL SaeVvkDLWP IERRRQREFF CFGMDILLKL DLpATRREFFD AFFDLePrYW 480
 TTKQINSN.I SRQAWDTLWP PERKRQRAFF LFGLALIVQF DTEGIRSFFR TFFRLPKVMW
 -----S---W--LWP -ER-RQR-FF -FG----- D----R-FF- -FF-L-----W
 ↑ CONSERVED REGION #5
 481 PLANT BETA
 A.t. EPSILON
 CONSENSUS
 HGFLLSSRLFL PELivFGLSL FShASNTSR- EIMTK.GI-P Lv-MINNLIQ D-e 533
 QGFLGSTLTS GDLVLFALYM FVISPNNLRK GLINHLISDP TGATMIKTYL KV.
 -GFL-S-L-- --L--f-L-- F----N-R- -----

PREDICTED TM HELIX

FIG.13B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/00540

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 67, 189, 193, 233, 252.3, 254.11, 320.1, 325, 419; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| A | CUNNINGHAM, JR. et al. Cloning and functional expression in <i>Escherichia coli</i> of a cyanobacterial gene for lycopene cyclase, the enzyme that catalyzes the biosynthesis of β -carotene. FEBS Letters. August 1993, Vol. 328, No. 1-2, pages 130-138. | 1-8 |
| X, P | CUNILLERA et al. <i>Arabidopsis thaliana</i> contains two differentially expressed farnesyl-diphosphate synthase genes. Journal of Biological Chemistry. 29 March 1996, Vol. 271, No. 13, pages 7774-7780. | 9-14, 27, 28, 30-32 |
| X, P | SUN et al. Cloning and functional analysis of the β -carotene hydroxylase of <i>Arabidopsis thaliana</i> . Journal of Biological Chemistry. 04 October 1996, Vol. 271, No. 40, pages 24349-24352. | 15-24 |

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

| | | |
|--|-----|--|
| * Special categories of cited documents: | * T | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| * A | | document defining the general state of the art which is not considered to be of particular relevance |
| * E | | earlier document published on or after the international filing date |
| * L | | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified) |
| * O | | document referring to an oral disclosure, use, exhibition or other means |
| * P | | document published prior to the international filing date but later than the priority date claimed |
| | * X | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| | * Y | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| | * A | document member of the same patent family |

Date of the actual completion of the international search

11 APRIL 1997

Date of mailing of the international search report

07 MAY 1997

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

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ERIC GRIMES

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/00540

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| A | BARTLEY et al. Molecular biology of carotenoid biosynthesis in plants. Annual Review of Plant Physiology and Molecular Biology. 1994, Vol. 45, pages 287-301. | 1-32 |
| A | GOODWIN. Biosynthesis of carotenoids: An overview. Methods in Enzymology. 1993, Vol. 214, pages 330-340. | 1-32 |
| A, P | US 5,589,581 A (MISAWA ET AL.) 31 December 1996, columns 1-3. | 1-32 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/00540

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☒

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/00540

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12N 1/21, 5/10, 9/02, 9/10, 9/90, 15/53, 15/54, 15/61, 15/63; C12P 23/00; C12Q 1/68

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/6, 67, 189, 193, 233, 252.3, 254.11, 320.1, 325, 419; 536/23.2

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Dialog, APS

search terms: IPP, isopentenyl pyrophosphate isomerase, epsilon cyclase, isopentenyl diphosphate isomerase, carotene hydroxylase, carotenoid, synthesis, biosynthesis, Arabidopsis thaliana, Haematococcus pluvialis

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claims 2-8, drawn to epsilon cyclase enzyme, DNA encoding epsilon cyclase, vectors and host cells comprising said DNA.

Group II, claims 9-14, drawn to isopentenyl pyrophosphate (IPP) isomerase enzymes, DNA encoding IPP isomerase, vectors and host cells comprising said DNA.

Group III, claims 15-24, drawn to beta carotene hydroxylase enzyme, DNA encoding beta carotene hydroxylase, vectors and host cells comprising said DNA.

Group IV, claims 25, 26, and 32, drawn to methods of screening using DNA comprising carotenoid biosynthesis genes.

Group V, claims 27, 28, 30, and 31, drawn to methods of using DNA encoding IPP isomerase.

Group VI, claim 29, drawn to a method of using antisense DNA.

Claim 1 is generic to Groups I, II, and III and will be examined with the elected Group(s) to the extent it reads thereon.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The claims of Group I share a technical feature of epsilon cyclase; the claims of Group II share a technical feature of IPP isomerase; the claims of Group III share a technical feature of beta carotene hydroxylase; the claims of Group IV share a technical feature of a screening method; the claims of Group V share a technical feature of methods of using DNA encoding IPP isomerase; and the claim of Group VI has a technical feature of antisense DNA. Carotenoid biosynthetic enzymes and genes were known in the art. See the references cited on page 3 of the disclosure; see also Spurgeon et al. (Arch. Biochem. Biophys. 230(2):446-454 (1984); IPP isomerase). Hence, the various Groups of inventions do not share a technical relationship involving one or more of the same or corresponding special technical features, i.e., those technical features that define a contribution which each invention, considered as a whole, makes over the prior art. They therefore do not fulfill the requirements of unity of invention and a holding of lack of unity for examination purposes is proper. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.